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Functional analysis of the extended N-terminus for the Drosophila Raf protein and initial characterization of the Arl1 gene

Jian Ding
Iowa State University

Functional analysis of the extended N-terminus for the Drosophila Raf protein and initial characterization of the Arl1 gene

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Jian Ding

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Program of Study Committee:
Linda Ambrosio, Major Professor
Amy Andreotti
Jack Girton
Ted Huiatt
Jo Anne Powell-Coffman

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ABSTRACT

The Raf serine/ threonine kinases play a key role(s) in receptor tyrosine kinase (RTK) signaling. However, the mechanisms that modulate Raf activity are complicated and remain elusive. Clues to Raf regulation were derived from the identification of conserved regions/motifs/sites. Drosophila Raf (DRaf) has an extended N-terminus. We show this N-terminal segment contains a novel region (CRN) that is conserved in BRaf proteins of vertebrates. The extended N-terminus can contribute to Torso RTK signaling during embryogenesis in both loss and gain-of-function genetic backgrounds. Furthermore, stronger interactions between DRaf's RBD (Ras Binding Domain) and the small GTPase Ras1, as well as Rap1, were observed *in vitro* when CRN and RBD sequences were linked. Together, these studies suggest that the N-terminal segment may assist in the association of DRaf with its activators (Ras1 and Rap1) and play a positive regulatory role(s) in DRaf activation *in vivo* through CRN-mediated mechanisms.

We also tried to characterize the roles of DRaf's conserved CR2 domain. In our yeast 2-hybrid screen using CR2 as the bait, a putative DRaf binding partner, small GTPase Arf-like1 (Arl1) protein, was identified. Using genetics approaches we have made an effort to understand the roles of Arl1 in Drosophila. Our studies suggest that Arl1 may function in membrane traffic.

CHAPTER 1. General Introduction

Introduction

Numerous fundamental cellular processes including differentiation, proliferation and cell survival in eukaryotes dependent on the Ras-Raf-MEK signal cassette and its regulation of extracellular signal-regulated kinase (ERK) pathways (SCHLESSINGER 2000). Binding of ligands with receptor tyrosine kinases (RTK) activates RasGEFs (Guanine-nucleotide exchange factors), which initiates GTP loading of Ras. GTP-Ras recruits Raf protein to the cell surface and triggers its activation. Once activated, Raf can phosphorylate its downstream effectors MEK (mitogen-activated protein and extracellular signal-regulated kinase kinase), subsequently leading to activation of ERK that modulates the transcriptional program of target genes (ROBBINS *et al.* 1994; Figure I-1). Dysregulation of ERK pathways caused by activating mutations in RTKs and components of the Ras-Raf-MEK module have been frequently found in human cancers (BEERAM *et al*. 2005; SRIKALA *et al.*, 2005). Therefore, study of the regulatory mechanisms of these ERK pathway complexes, which represent the strategic targets of anti-tumor drugs, will be very essential for development of therapeutics against cancer.

Ras has been evaluated as a putative target of anti-cancer therapeutic strategies, since oncogenic mutations in Ras were linked to ~15% human cancers (WELLBROCK *et al*. 2004). Both the regulatory mechanisms (GTPase cycle, post-translational modification), as well as the biophysical nature of Ras and other small GTPases in general have been relatively well defined. However, the clinical results of Ras-targeting drugs, including farnesyltransferase

(FTase) inhibitors (FTIs) *etc*, appear disappointing (ROWINSKY *et al.* 1999; BEERAM *et al*. 2005; SRIKALA *et al.* 2005).

As a major effector of Ras, the Raf serine/ threonine kinases play a key role in ERK signal transduction. Constitutively active forms of Raf and Ras appear to have a comparable potential to transform malignant cells, while expression of dominant negative mutants of Raf can antagonize Ras transforming activity. Furthermore, it has been discovered that BRaf is mutated at a high frequency in human cancers, particularly in 70-80% malignant melanomas (Reviewed by SRIKALA *et al.* 2005). These observations have led to considerable amount of attention paid to targeting Raf as an anticancer therapeutic strategy, thus highlighting the importance of Raf kinases. Therefore, understanding of the mechanisms that modulate Raf activity is an important issue with physiological significance.

Mammals have three Raf isoforms, ARaf, BRaf and CRaf, while the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* have one *Raf* gene referred to as *Lin-45* and *DRaf*, respectively. All the Raf proteins share a common primary structure consisting of three conserved regions (CR1, CR2 and CR3, Figure I-2). Clues to regulatory events of Raf were derived from the identification of motifs/sites in these conserved regions. The catalytic portion of the kinase is located in the C-terminal conserved region 3 (CR3), while CR1 and CR2 represent the regulatory half of the Raf protein. Although an auto-inhibitory role had been previously assigned to residues compromising CR1 and CR2, the N-terminal regulatory part also functions in promoting Raf activity. CR1 contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and is required for recruitment of Raf to the plasma membrane. The Ser/Thr-rich region CR2 has a 14-3-3 binding site, but its functional role is less well defined (WELLBROCK *et al*. 2004). In addition to CR1, CR2 and CR3, BRaf

and DRaf contain an extended N-terminal segment followed by CR1. However, little attention was given to this region thus far.

Research in our laboratory has focused on understanding the regulatory mechanisms of the DRaf protein in the model system Drosophila. Particularly, our goal is to characterize the roles of the extended N-terminal segment and CR2 region in DRaf signaling. We have found that the N-terminus of DRaf contains a novel region that is conserved in BRaf proteins of vertebrates. It may assist in Ras-DRaf interaction and can play a positive role(s) in Torso RTK signal transduction during *Drosophila* embryogenesis. We also discovered a putative DRaf binding partner, small GTPase Arf-like1 (Arl1) protein, which interacts with CR2 region. Using genetics approaches we have made an effort to understand the functional roles of Arl1 in Drosophila.

Literature Review

Activation of Raf by Ras: Cytosolic Raf proteins exist as an "inactive" complex with other binding partners including 14-3-3 and heat shock protein (Hsp90). Raf has two specific serine residues that act in 14-3-3 binding and lie within the CR2 (Ser259, refer to CRaf) and CR3 (Ser621, refer to CRaf) domains, respectively. Association of either site with 14-3-3 requires phosphorylation of the corresponding serine residues. Formation of the inactive complex via the interaction between 14-3-3 and both of these phospho-Ser residues may maintain the Raf molecule in a "closed" conformation and mask its catalytic region, since a Ser259 to Ala mutation that may prevent the CR2 from interacting with 14-3-3 appears to facilitate CRaf activation (CLARK *et al*. 1997; KUBICEK *et al.* 2002).

Activation of Raf is initiated by its translocation to membrane, where Raf can form a complex with different binding partners to elicit its full activation. The small GTPase Ras plays a crucial role(s) in regulation of Raf's membrane localization and is evaluated as a major regulator of Raf, although it was reported that interaction between a basic motif in CRaf's kinase region and phosphatidic acid (PA) can recruit Raf to the membrane in a Ras-independent way (RIZZO *et al*. 2000; KRAFT *et al.* 2008). Association with Ras, not only translocates Raf to the membrane, but may disrupt 14-3-3 binding to CR2, leaving the phospho-Ser259 residue more accessible to dephosporylation by protein phosphatase 2A (PP2A) or other phosphatases (KUBICEK *et al.* 2002). However, the molecular mechanisms of Ras-Raf coupling is complicated and not completely defined.

Cellular Ras exists in two guanine nucleotide-bound states, GTP-Ras and GDP-Ras, with different molecular conformations in its switch 1 and switch 2 regions. Raf's RBD can directly interact with GTP-Ras and is thought to be the core element for Ras-binding (NASSAR *et al*. 1995). Arg89 (refer to CRaf) in RBD is essential for its association with Ras and substitution of Arg 89 to Leu abolishes this interaction (FABIAN *et al*. 1994; LI *et al*. 1998). In addition, CRD functions in Ras-Raf coupling, as well, through its contact with the lipid moiety of Ras, which is independent of GTP status of Ras. It seems the hydrophobic patch within CRD is required for Ras binding, since mutation of the hydrophobic surface impairs the interaction (WILLIAMS *et al*. 2000; THAPAR *et al*. 2004). Recently, it was found that BRaf's extended N-terminus facilitated BRaf-HRas interaction *in vitro*, and deletion of the N-terminal segment reduced BRaf' s binding affinity with HRas (FISCHER *et al.* 2007. However, the identities of residues/sites in this process were not characterized.

Phosphorylation of Raf: Phosphorylation/dephosphorylation of Raf plays crucial roles in Raf regulation. Identification of conserved phosphorylation sites/motifs has provided very important clues in understanding Raf's regulatory mechanisms.

Phosphorylation of Ser43, Ser259 and Ser233 (refer to CRaf with Ser43 and Ser233 specific for CRaf) exhibits negative effects on Raf activation. Phosphorylation of Ser43 both *in vivo* and *in vitro* can reduce the apparent Ras binding affinity and appears to sterically prevent the interaction of CR1 with Ras (WU *et al.* 1993). In CR2 region, phosphorylated Ser259 mediates the interaction with 14-3-3 (CLARK *et al*. 1997). It was reported that phosphorylation of Ser233 provides an additional binding site for 14-3-3 (DUMAZ and MARAIS 2003). Therefore, phosphorylated Ser 259 and Ser233 may contribute together to maintain the Raf molecule in an inactive conformation. These three serine residues (Ser43, Ser259 and Ser233) seem to be phosophorylated by protein kinase A (PKA) when the cellular cAMP level is elevated (DUMAZ *et al.* 2002). It was also found that Ser259 can be phosphotylated by AKT (also known as protein kinase B, PKB) and serum glucocorticoid-inducible kinase (SGK) (ZIMMERMANN and MOELLING 1999; BEERAM *et al*. 2005)

The C-terminal phosphorylated Ser621 (Refer to CRaf) is another 14-3-3 binding site and seems to play dual roles, both negatively and positively, in Raf activation. 14-3-3 associates with both phospho-Ser259 and Ser621, and helps to maintain the inactive Raf complex. However, when its binding to Ser259 is disrupted, the interaction with phospho-Ser621 seems essential to attain full activation of Raf, since mutation at Ser621 abolishes Raf's activity in cultured Cos-7 cells (YIP-SCHNEIDER *et al.* 2000). At the present time, it is still controversial whether phosphorylation of Ser621 is regulated during Raf activation.

The N-region (Negatively charged) preceding the catalytic kinase domain in CRaf contains two additional regulatory sites Ser388 and Tyr341. Phosphorylation of these two residues seems to relieve the inhibitory effects of the N-terminal regulatory portion of the protein on its catalytic domain and is required to achieve full activation of the kinases. Mutations at either site block CRaf activation, and maximal activity is not obtained unless both sites are phosphorylated (MASON *et al*. 1999). Phosphorylation of Ser338 and Tyr341 in CRaf is likely mediated by PAK and Src family kinases, respectively (KING *et al.* 1998; TILBROOK *et al*. 2001).

A few of these phosphorylation sites mentioned above are isoform specific. The two conserved residues (Thr491, Ser494, refer to CRaf) residing in the active segment within the kinase domain are probably common to all Raf members (DOUZIECH *et al.* 2006). Phosphorylation of these two sites seems essential for activity of Raf family members with their mutations abolishing Raf activation (CHONG *et al.* 2001). However, the identity of those kinases, which mediate this phosphorylation, remains unknown.

Recently, a novel phosphorylation site (Tyr510) in DRaf was identified. This Tyr residue is conserved in all Raf proteins. Phosphorylation of this Tyr510 by Src68B kinase appears to relieve autoinhibition of full-length DRaf in Drosophila (XIA *et al.* 2008). This regulatory mechanism may be shared by other Raf proteins, including ARaf, BRaf and CRaf.

Regulation by scaffold proteins and molecular adaptors: Raf's activity is also modulated through formation of functional complexes with scaffolding adaptors, including the kinase suppressor of Ras (KSR) and connector enhancer of KSR (CNK).

KSR contains a C-terminal kinase-like domain, which interacts with MEK. However, this domain, in which the key sites essential for kinase function are substituted with other

residues. appears to be devoid of catalytic kinase activity. In stead, it has been widely depicted that KSR acts as a scaffold protein rather than kinase, and coordinates the assembly of Raf-MEK-MAPK complexes (ROY *et al.* 2002; ROY and THERRIEN 2002; ROIGNANT *et al.* 2006; RAJAKULENDRAN *et al.* 2008). Recently, it was found that overexpression of KSR without co-overexpression of other scaffolding partners in Drosophila S2 cells could independently trigger DRaf activation, probably by participating in heterodimmerization with DRaf in a side-to-side manner. This suggests that beyond its "scaffolding" function, KSR may possess an intrinsic Raf activating potential (RAJAKULENDRAN *et al.* 2009).

CNK does not contain any catalytic regions/motifs. Instead, CNK has several conserved domains, which provide numerous interaction sites for its binding partners, suggesting it functions as a scaffold protein (KOLCH 2005). Drosophila CNK plays dual roles in DRaf regulation, both negatively and positively, according to RTK signal status. Its C-terminal RIR (Raf inhibitory region) interacts with DRaf and inhibits Raf activity in quiescent S2 cells. However, once RTK is activated, the N-terminal SAM (sterile α-motif) and CRIC (a conserved region in CNK) regions mediate Raf activation by Ras, and the inhibitory effects of RIR can be relieved by active Src42 (DOUZIECH *et al*. 2003; DOUZIECH *et al.* 2006). The RIR is not conserved in CNK proteins of mammals. Mammalian CNK can promote CRaf activation by simultaneously interacting with CRaf and Src, thereby facilitating the phosphorylation of Tyr341 in CRaf by Src (LANIGAN *et al*. 2003; KOLCH 2005).

Isoform-specific regulation of Raf kinases: All the Raf members have a similar primary structure, share Ras GTPase as a common regulator, MEK as the common effector, and were thought activated by similar means. However, differences in their regulatory mechanisms have recently emerged.

It has been reported that RBDs of different Raf isoforms differentially interact with active Ras. BRaf and CRaf RBD's exhibit higher binding affinity with HRas than ARaf RBD *in vitro*, and consistently, in HEK 293 cells, active HRas preferentially activates CRaf as compared to ARaf. The isoform specific Ras binding affinity is, at least partially, due to one residue difference in their RBD regions, Arg59 of CRaf is replaced with Lys (Lys22) in ARaf, since mutation of Lys22 to Arg in ARaf can increase its affinity to HRas *in vivo* and *in vitro* (WEBER *et al*., 2000).

Both BRaf and CRaf can interact with the small GTPase Rap, which shares nearly identical effector binding portions with Ras. However, association of different Raf isoforms with active Rap results in distinct effects in signaling. Rap can activate BRaf in a parallel way with Ras, but plays a negative role and functions as an antagonist of Ras in regulating CRaf activity. It was found that CRD of CRaf exhibits higher binding affinity for Rap than that of BRaf. The strong interaction between CRaf's CRD and Rap may exclude Ras binding, therefore inhibiting activation of CRaf (OHTSUKA, *et al.* 1996). However, it is still unclear why CRaf and BRaf are regulated by Rap in distinct manners.

Furthermore, modulation of Raf kinases' phosphorylation status appears to be isoform-specific, too. The Ser43 site in CRaf is not conserved in ARaf or BRaf. BRaf contains two specific phosphorylation sites (Ser428 and Thr439) that ARaf and CRaf do not possess. Phosphorylation of Ser338 in CRaf, which is critical for its activation, is responsive to upstream stimulation, while the corresponding Ser445 in BRaf is constitutively phosphorylated. In the regulatory N-region preceding the kinase domain, BRaf lacks a tyrosine site corresponding to Tyr301 of ARaf and Tyr341 of CRaf, instead, it has an acidic residue (Asp448) in the equivalent position. To obtain full activation, Tyr301 of ARaf and

Tyr 341 of CRaf need to be phosphorylated by Src. However, for BRaf, the corresponding acidic Asp448 residue mimics consititutive phosphorylation and results in a higher basal activity for Braf (MASON *et al*., 1999).

Endogenous Raf proteins are predominantly distributed in the cytoplasm. However, overexpressed Raf kinases exhibit isoform specific intracellular localization. CRaf was found located in mitochondria, while ARaf was colocalized with an endosomal marker (GALMICHE *et al.* 2008). The different cellular localization patterns suggest these Raf members play isoform specific roles, although originally they were thought to be functional redundant to some extent.

BRaf isoform specific features: Compared with ARaf and CRaf, BRaf has a higher basal activity and is more liable to oncogenic mutation. Somatic mutations of BRaf were frequently found in different human cancers (~83%anaplastic thyroid carcinoma, and 55-68% malignant melanoma). Particularly, a substitution of Val599 with Glu ($BRaf^{V599E}$) accounts for ~90% oncogenic BRaf mutations in cancers (SRIKALA *et al.*, 2005). $BRaf^{V599E}$ is constitutively active, and its activity appears to be independent of Ras binding. Crystal structure of BRaf kinase doman indicates that the V599E mutation may eliminate the requirement for activation segment phosphorylation. Surprisingly, some BRaf mutants with impaired kinase activity can activate CRaf and signal to ERK in cells, suggesting that BRaf may function as a scaffold protein (WAN *et al.* 2004). Furthmore, over-expression of wild type BRaf in malignant melanoma without NRas mutation can activate MAPK signal and promote cell growth (TANAMI *et al*. 2004), indicating that BRaf may have an intrinsic potential to activate CRaf (or other BRaf molecules). These studies highlight the importance and physiological significance of BRaf. Thus, understanding isoform-specific functions and

regulatory mechanisms of BRaf has been attracting more research interest.

The negative charged motif (D447-D448) preceding the kinase domain is a BRaf specific feature. As mentioned already, the corresponding sites in ARaf and CRaf must be phosphorylated by Src kinases to achieve full activation. However, the negative charged motif in BRaf mimics constitutive phosphorylation and may account for BRaf's high basal activity (OHTSUKA, *et al.* 1996; MASON *et al*. 1999).

Different from ARaf and CRaf, BRaf has an extended N-terminus in addition to CR1, CR2 and CR3. However, studies of BRaf regulation have mainly focused on CR1, CR2 and CR3 with little attention, thus far, given to the role of this BRaf specific N-terminal region. Although Fischer *et al.* (2007) found this extended N-terminus facilitated BRaf's interaction with HRas *in vitro*, at the present time, the molecular mechanisms are not clear and the biological implications of this BRaf specific N-terminal region *in vivo* are not well defined.

Drosophila Raf: Drosophila has one *Raf* gene encoding DRaf protein. As a key component of the MAP kinase signaling module, DRaf plays an essential role in numerous receptor tyrosine kinase (RTK) pathways in Drosophila development. The regulatory mechanisms of DRaf are quite similar to that of mammalian Raf proteins. Almost all the regulators, co-factors as well as effectors of DRaf are conserved in mammals. Forward genetics screen for components of Ras-Raf-MAPK pathway in Drosophila have tremendously advanced our understanding of the signaling in mammals, and study of DRaf have been providing lots of important clues regarding to regulator mechanisms of mammalian Raf proteins.

Based on the primary structures, Drosophila Raf is more similar to BRaf than either ARaf or CRaf. DRaf has 61% overall sequence similarity with BRaf. DRaf shares isoform

specific features with BRaf and is thought to be a BRaf ortholog. DRaf has the signature negative charged motif (E420-E421) that corresponds to D447-D448 within BRaf (MASON *et al*. 1999; MISHRA *et al.* 2005). DRaf also has an extended amino-terminus followed by CR1. DRaf and BRaf also share parallels in their modes of regulation. Rap1 can activate both BRaf and DRaf, but not ARaf or CRaf (MISHRA *et al.* 2005; OHTSUKA, *et al.* 1996). Therefore, studies on DRaf using the powerful Drosophila genetic system may provide important unique insights to better understand the functional roles and regulatory mechanisms of BRaf.

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Figure Legends

FIGURE I-1. Raf-mediated Receptor Tyrosine Kinase (RTK) signal pathways: The binding of ligand to a RTK receptor triggers the activation of small GTPase Ras, which recruits Raf to the plasma membrane. Once activated, Raf transfers its signal to MEK, in turn leading to activation of ERK. By regulating downstream gene expression, the RTK pathways function in numerous fundermental processes, including differentiation, proliferation, cell survival and cell fate determination.

FIGURE I-2. Schematic representations of Raf kianses: Raf family members share a common primary structure consisting of three conserved regions (CR1, CR2 and CR3). CR1 contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and is required for recruitment of Raf to the plasma membrane. The Ser/Thr-rich region CR2 has a 14-3-3 binding site. CR1 and CR2 represent the regulatory half of the Raf protein, while the catalytic portion of the kinase is located in the C-terminal conserved region 3 (CR3). In addition to CR1, CR2 and CR3, BRaf and DRaf contain an extended N-terminal segment followed by CR1.

FIGURE I-1

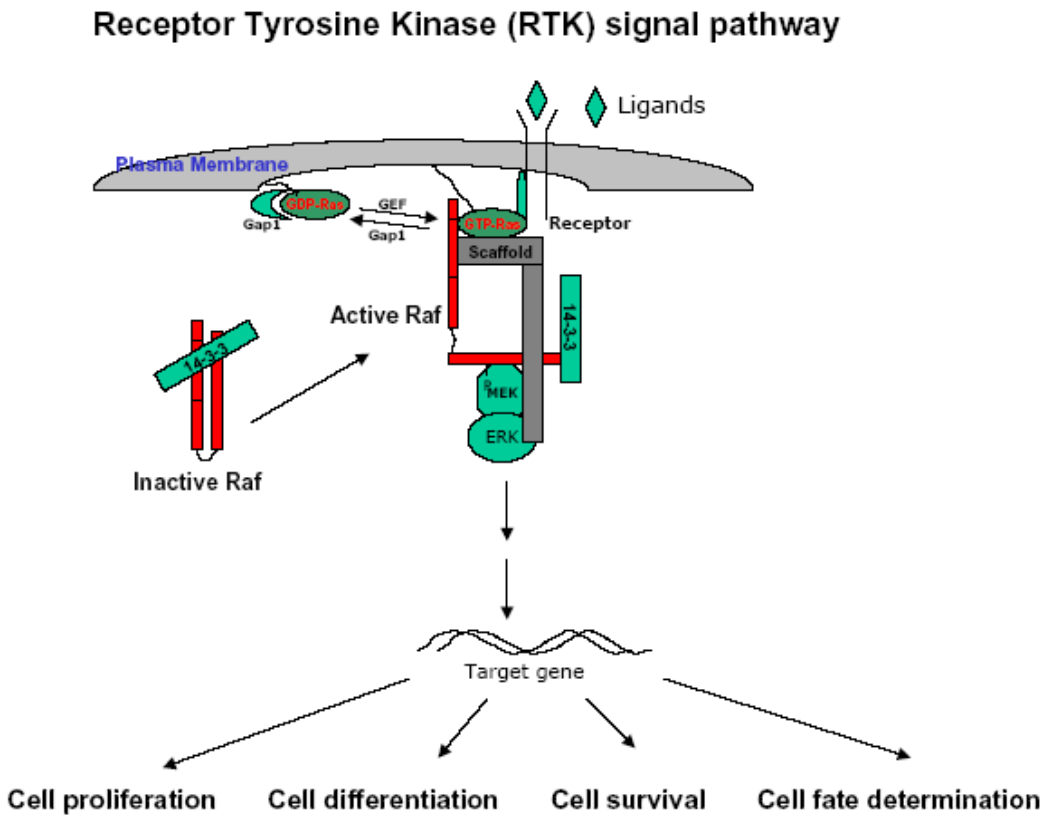
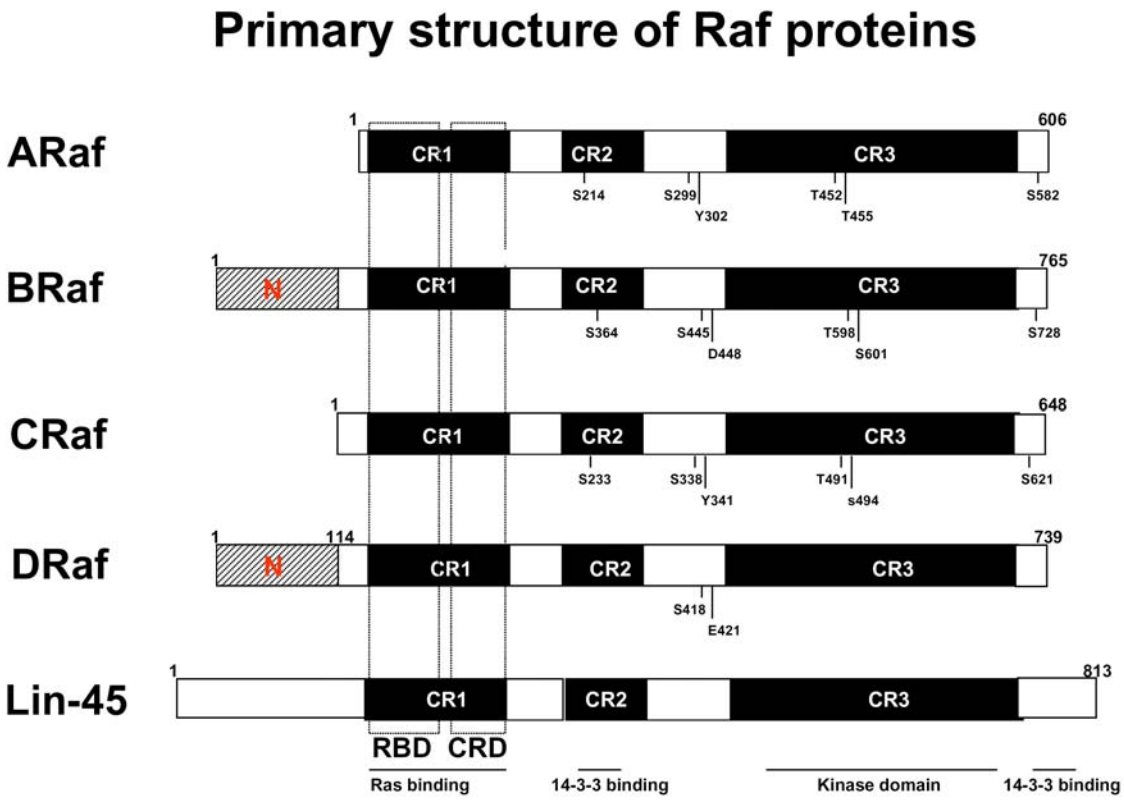

Receptor Tyrosine Kinase (RTK) signal pathway
Ligands
Plasma Membrane
GDP-Ras
GEF
Gap1
Gap1
GTP-Ras
Receptor
Scaffold
Active Raf
14-3-3
14-3-3
P
MEK
ERK
Inactive Raf
Target gene
Cell proliferation
Cell differentiation
Cell survival
Cell fate determination

FIGURE I-2


Primary structure of Raf proteins
ARaf
1
606
CR1
CR2
CR3
S214
S299
Y302
T452
T455
S582
BRaf
1
765
N
CR1
CR2
CR3
S364
S445
D448
T598
S601
S728
CRaf
1
648
CR1
CR2
CR3
S233
S338
Y341
T491
s494
S621
DRaf
1
114
739
N
CR1
CR2
CR3
S418
E421
Lin-45
1
813
CR1
CR2
CR3
RBD
CRD
Ras binding
14-3-3 binding
Kinase domain
14-3-3 binding

CHAPTER 2. Drosophila Raf's N-terminus Contains a Novel Conserved Region and Can Contribute to Torso RTK Signaling

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Jian Ding, Oren Tchaicheeyan and Linda Ambrosio*
Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa, 50011
*Corresponding author (lima@iastate.edu)

Abatract

Drosophila Raf (DRaf) contains an extended N-terminus, in addition to three conserved regions (CR1-3), however, the function(s) of this N-terminal segment remains elusive. In this paper, a novel region within DRaf's N-terminus that is conserved in BRaf proteins of vertebrates was identified and termed Conserved Region N-terminal (CRN). We show that the N-terminal segment can play a positive role(s) in the Torso receptor tyrosine kinase pathway *in vivo*, and its contribution to signaling appears to be dependent on the activity of Torso receptor, suggesting this N-terminal segment can function in signal transmission. Circular dichroism analysis indicates that DRaf's N-terminus (amino acids 1-117) including CRN (amino acids 19-77) is folded *in vitro* and has a high content of helical secondary structure as predicted by proteomics tools. In yeast two-hybrid assays, stronger interactions between DRaf's Ras binding domain (RBD) and the small GTPase Ras1, as well as Rap1, were observed when CRN and RBD sequences were linked. Together, our studies suggest that DRaf's extended N-terminus may assist in its association with the upstream activators (Ras1 and Rap1) through a CRN-mediated mechanism(s) *in vivo*.

Introduction

Evolutionarily conserved receptor tyrosine kinase (RTK) signaling pathways function in fundamental cellular processes including differentiation, proliferation and cell survival in eukaryotes (SCHLESSINGER 2000). The Raf serine/threonine kinase, as a key component of RTK signaling modules, plays a central role in transmitting upstream stimuli to the nucleus (DAUM *et al.* 1994). Cyclic control of Raf depends on activities of GTPases, kinases, phosphatases and scaffold proteins (CHONG *et al.* 2001; DHILLON *et al*. 2002; KOLCH 2000; MORRISON 2001; RAABE and RAPP, 2002). Clues to these regulatory events were derived from the identification of conserved regions/motifs/sites. However, the mechanisms that modulate Raf serine/threonine kinases are complicated and remain elusive.

Mammals have three Raf isoforms, ARaf, BRaf and CRaf. They share a similar primary structure consisting of three conserved regions (CR1, CR2 and CR3). Conserved region 1 (CR1), where a Ras binding domain (RBD) and a cysteine-rich domain (CRD) reside, is required for Ras-Raf interaction. CR2, a serine/threonine-rich region, contains a 14-3-3 binding site. CR1 and CR2 are embedded in the regulatory N-terminal half of Raf proteins, while CR3, including the catalytic kinase region and an additional 14-3-3 binding site, resides in the C-terminus (Reviewed by WELLBROCK *et al*. 2004). In addition to these three conserved regions, BRaf has an extended amino-terminal segment followed by CR1 (TERAI and MATSUDA 2006; FISCHER *et al*. 2007). However, studies of BRaf regulation have mainly focused on CR1, CR2 and CR3 with little attention, thus far, given to the role of this N-terminal region.

Translocation of Raf proteins to the plasma membrane, a critical step in their activation, can be mediated through different mechanisms. It is reported that direct interaction between a

basic motif in CRaf's kinase region and phosphatidic acid (PA) can recruit Raf to the membrane (RIZZO *et al*. 2000; KRAFT *et al.* 2008). This PA-binding site is conserved in ARaf and BRaf proteins. Also, association with Ras, a major regulator of Raf kinases, plays a crucial role(s) in translocation and activation of Raf. However, the molecular mechanisms of Ras-Raf coupling are not completely understood. Raf's RBD can directly interact with the switch 1 region of GTP-Ras and is thought to be the core element for Ras binding (NASSAR *et al*. 1995). CRD is involved in Ras-Raf coupling, as well, through interaction between its hydrophobic patch and the lipid moiety of Ras (WILLIAMS *et al*. 2000; THAPAR *et al*. 2004). Thus, both RBD and CRD contribute to Ras-Raf interaction and the effects are likely additive. Disabling either RBD or CRD is thought to reduce but not completely eliminate Raf activity (HU *et al.* 1995). Recently, Fischer *et al.* (2007) found BRaf's interaction with HRas was also facilitated by the extended N-terminus, *in vitro*. At the present time, however, the identity of residues/sites that participate in this process are unknown and the biological implications of this N-terminal region *in vivo* have not been defined.

Drosophila has one *Raf* gene first described genetically as *l(1) pole hole*, and later referred to as *DRaf* or *Raf*. As a member of the MAP kinase signaling module, DRaf plays an essential role in numerous receptor tyrosine kinase (RTK) pathways in Drosophila development (BRENNAN and MOSES 2000; VAN BUSKIRK and SCHÜPBACH 1999; DUFFY and PERRIMON 1994; RAABE 2000). Based on its primary structure, the DRaf protein is more similar to BRaf than either ARaf or CRaf (DHILLON and KOLCH 2002; CHONG *et al.* 2003; MORRISON and CUTLER 1997). DRaf and BRaf have two acidic residues (E420-E421 in DRaf; D447-D448 in BRaf) preceding the kinase region that correspond to residues Y301-Y302 in ARaf and Y340-Y341 in CRaf, respectively. These negative charged acidic residues

mimic constitutive phosphorylation and are thought to be related to the higher basal activity of BRaf (MASON *et al*., 1999; MISHRA *et al.* 2005). Both DRaf and BRaf have an extended amino-terminus, when compared to ARaf and CRaf, in addition to CR1, CR2 and CR3. DRaf and BRaf also share parallels in their modes of regulation. Rap1 can activate both BRaf and DRaf, but not ARaf or CRaf (MISHRA *et al.* 2005; OHTSUKA, *et al.* 1996). Like the Raf proteins in mammals, the activity of DRaf is regulated through phosphorylation/dephosphorylation (BAEK *et al.* 1996; LABERGE *et al.* 2005; RADKE *et al.* 2001; ROMMEL *et al.* 1997), interaction with scaffold proteins or other binding partners (DOUZIECH *et al*. 2003; DOUZIECH *et al.* 2006; ROY *et al.* 2002; ROY and THERRIEN 2002; ROIGNANT *et al.* 2006; RAJAKULENDRAN *et al.* 2008). These regulatory events occur within the three conserved regions (CR1-3) of DRaf, however, the role of DRaf's N-terminal region has not been elucidated.

Development of both embryonic termini in Drosophila is dependent on DRaf-mediated Torso RTK signaling. Binding of Trunk or Torso-like with the Torso receptor initiates Ras1-DRaf-MEK signaling at the poles of early staged embryos, and in turn, triggers expression of at least two gap genes, *tailless* and *huckebein*, which specify terminal structures and help to establish segmental identities in the embryo (Reviewed by FURRIOLS and CASANOVA 2003). The domain of *tailless* (*tll*) expression in the embryonic posterior region has been used as a quantitative marker to measure the strength of the Torso RTK signal in early embryos. At the cellular blastoderm stage, embryos from wild type mothers show posterior *tll* expression from approximately 0-15% embryo length (EL). At a later stage embryos exhibit normal internal head structures, three thoracic segments (T1-T3), eight abdominal denticle belts (A1-A8), as well as the Filzkörper tail structure. Decreased or loss of Torso RTK pathway activity

results in a reduced posterior expression domain of *tll* and consequently absence of embryonic tail structures. In contrast, gain-of-pathway activity can lead to expanded *tll* expression domains at both poles, and subsequently enlarged head and tail structures, accompanied by deletion of central abdominal segments (GHIGLIONE *et al*. 1999; JIMÉNEZ *et al.* 2000).

In this study, using the Drosophila embryonic termini as both a qualitative and quantitative *in vivo* assay system, we examined the role played by DRaf's N-terminus in Torso signaling in different genetic backgrounds. We observed a subtle, but consistent, higher signaling potential for full-length DRaf proteins when compared with those lacking amino-terminal residues 1-114 ($DRaf^{\Delta N114}$). Furthermore, a novel region within DRaf's N-terminus that is conserved in RAF genes of most invertebrates and BRaf genes of vertebrates was identified and termed Conserved Region N-terminal (CRN). Our studies suggest that DRaf's extended N-terminus may assist in its association with the upstream activators (Ras1 and Rap1) *in vivo* and thus, potentially play a regulatory role(s) in DRaf's activation through a CRN-mediated mechanism(s). Minor adjustment by CRN on Ras1 and Rap1 binding may help to fine-tune DRaf's activity and consistently provide optimal signal output.

Materials and Methods

Drosophila strains and genetics: In this study, *y w*, $Draf^{11\text{-}29}$ ($Draf^{-}$; DRaf protein null, MELNICK *et al.* 1993), $trunk^{1}$ (trk^{-}; loss-of-function allele, lacks C-terminal 16 amino acids, SCHÜPBACH and WIESCHAUS 1989, CASANOVA *et al.* 1995), $torso^{XR1}$ (tor^{-}; Torso protein null allele, *tor* gene deletion, SPRENGER *et al.* 1989), and $torso^{RL3}$ (tor^{RL3}; gain-of-function allele, H242L amino acid replacement in the extracellular domain, SPRENGER *et al.* 1993)

strains were used. The "FLP-DFS" technique was utilized to generate *Draf* $^{11-29}$ germline clones (CHOU and PERRIMON 1996). Drosophila stocks were raised at 25° on standard cornmeal medium. To study the gain-of-function effects of the temperature-sensitive *tor*^{RL3} allele (Figure II-3), virgin females were collected and mated with wild type males at 25° for 3-4 days and then moved into a 29° incubator. Eggs were collected at 29° during the first 1-2 days for western analysis and phenotypic characterization.

Transgene design: Full-length and truncated DNAs were amplified using wild type DRaf cDNA (GenBank#AY089490, obtained from Drosophila Genomics Research Center) as template, and inserted into the polylinker site of the *P*-element transformation vector pCaSpeR-HS83. The full-length cDNA sequence (FL DRaf) encodes a DRaf protein with 739 residues, while the truncated cDNA sequence (DRaf$^{\Delta N114}$) corresponds to amino acid 115-739 of the FL DRaf protein. The constitutively active heat-shock 83 gene (HS83) promoter was used to drive the expression of DRaf transgenes to simplify the generation of transgenic lines with various genetic backgrounds. Transgenic lines were generated by Genetic Services (Sudbury, MA).

Multiple lines derived for each transgene were used in this study. DRaf$^{\Delta N114}$ (L1, #a and #b), FL DRaf (#a and #b) were used to generate germline clone bearing females (Figure II-1). Lines #1, #2, #3 of DRaf$^{\Delta N114}$ and #1, #2, #3 of FL DRaf were used in *tor*^{RL3}, *trk*^{1} and *tor*^{XR1} backgrounds. The DRaf$^{\Delta N114}$ line #1 is homozygous lethal, thus we generated *trk*^{1}*/trk*^{1}*; DRaf*$^{\Delta N114}$*#1/ DRaf*$^{\Delta N114}$*#3* and *tor*^{XR1}*/tor*^{XR1}*; DRaf*$^{\Delta N114}$*#1/DRaf*$^{\Delta N114}$*#3* lines that produce DRaf protein levels equivalent with other lines (Figure II-4, Table II-1).

Western analysis: To produce protein extracts, 100 eggs were collected and homogenized in 36ul lysis buffer containing 20mM Tris-Cl (pH8.0), 150mM sodium

chloride, 0.2% Triton-X 100, 0.2% Nonidet P-40, 10mM EDTA, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride, 0.15U/ml aprotinin and 20mM leupeptin. Insoluble material was removed by centrifugation (13000 rpm, 10 minutes) at 4 °C. Protein extracts were separated by 8% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. DRaf proteins were probed with rabbit anti-Raf antibody (70.1, SPRENGER *et al.* 1993) and horseradish peroxidase-coupled goat anti-rabbit secondary antibody (Thermo Scientific). α-tubulin proteins probed with mouse antibody (Sigma) and horseradish peroxidase-coupled goat anti-mouse secondary antibody (Thermo Scientific) were used as an internal control. The membranes were developed using SuperSignal West Pico kit (Thermo Scientific). Protein level was quantified with Image J.

***In situ* hybridization:** *tailless* and *engrailed* probes were generated from wild type cDNA clones (*tailless*: Genebank #BT022195; *engrailed*: Genebank #AY069448, obtained from the Drosophila Genomics Research Center) using the PCR DIG probe synthesis kit (Roche Applied Science). Whole-mount mRNA *in situ* hybridizations were performed in embryos according to the protocol of Tautz and Pfeifle (1989) with minor modifications.

Circular dichroism (CD) spectral measurement: DNA corresponding to amino acids 1-117 of DRaf ($DRaf^{N117}$) was recombined into the pGEX vector. The GST-$DRaf^{N117}$ fusion protein was produced by expression in *E.coli* BL21, and purified by standard affinity chromatography. Purified GST-$DRaf^{N117}$ protein was digested with thrombin. The $DRaf^{N117}$ protein (~13KD) was purified by a size-exclusive column (Amersham Biosciences), and verified by Mass spectra and N-terminal sequencing. Protein sample (0.05 mg/ml in 10 mM sodium phosphate buffer) was loaded to 0.1 cm quartz CD cuvette. CD spectra was measured by Jasco J-710 spectropolarimeter (Protein facility at Iowa State University) at room

temperature. Data were collected with 0.2 nm resolution and at a scan rate of 1.5 nm min^{-1}. The ellipcity value of the blank buffer at each wavelength was substracted from each point.

Yeast two-hybrid analysis: The R174 to L mutation in DRaf ($DRaf^{R174L}$) was generated by PCR-based site-directed mutagenesis, and confirmed by sequencing. DNA sequences corresponding to amino acid 1-117 (N), 1-212 (NRBD), 18-212 (Δ17NRBD), 78-212 (Δ77NRBD) and 115-212 (RBD) were obtained by PCR using wild type DRaf as the template, while DNAs encoding $NRBD^{R174L}$ and RBD^{R174L} were amplified from $DRaf^{R174L}$ DNA. Amplified DNAs were cloned into pGADT7 vector (Clontech). DNA sequences encoding amino acid 1-183 of Ras1 (Ras1ΔCAAX), 1-180 of Rap1 (Rap1ΔCAAX) were amplified from cDNAs of wild type Ras1 and Rap1 (Ras1: Genebank #AF186648; Rap1: NCBI Reference #NM_057509, obtained from the Drosophila Genomics Research Center) respectively, and inserted into the pGBKT7 vector (Clontech).

Constructed pGADT7 and pGBKT7 plasmids were transformed into yeast Y187 strain. Protein-protein interactions were tested by ß-Galactosidase assays using X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma; solid-support assay) or ONPG (ortho-nitrophenyl-b-D-galactopyranoside, Sigma; liquid quantitative assay) as substrates. ß-Galactosidase units in quantitative assays were calculated according to the Yeast Protocol Handbook (Clontech). All yeast two-hybrid experiments are confirmed by reciprocal bait-prey assays and repeated at least 4 times.

Results

To study the potential function of DRaf's N-terminal residues (amino acids 1-114), we generated transgenic flies expressing full-length DRaf (FL DRaf) or DRaf proteins lacking

amino-terminal residues 1-114 (DRaf$^{\Delta N114}$; Figure II-1A). The constitutive heat-shock 83 (HS83) promoter was selected to drive transgene expression, to simplify the generation of complex genetic backgrounds required to test the functionality of N-terminal residues. We used the Torso pathway to test the signaling potential of these maternally expressed DRaf proteins. Since the Torso signaling system is solely dependent on activity of maternal DRaf proteins, we could readily determine and verify the quantity of DRaf proteins available for Torso signal transduction in early staged embryos by western blot analysis. Thus, at equivalent protein concentrations, we compared the signal potential of FL DRaf and DRaf$^{\Delta N114}$ proteins to characterize the role of the N-terminus in a well-defined RTK pathway *in vivo*.

DRaf's N-terminus can contribute to RTK signaling in Drosophila embryos: Embryos that were deficient for maternal DRaf protein (derived from *Draf$^{11-29}$/Draf$^{11-29}$* female germ cells, see MATERIALS and METHODS) lack posterior *tll* expression at ~2.5 hours after egg deposition and subsequently exhibit abnormal cuticle pattern with deletion of posterior structures due to loss of Torso RTK signaling (Figure II-1D ii, ii'). We generated females with germ cells homozygous mutant for the *Draf$^{11-29}$* allele (*Draf^{-}/$^{-}$*) but expressing FL DRaf or DRaf$^{\Delta N114}$ proteins using the "FLP-DFS" technique (CHOU and PERRIMON, 1996). Cuticles of embryos produced by *Draf $^{-}$/$^{-}$; DRaf$^{\Delta N114}$* female germline clones expressing maternal truncated proteins at ≥ to endogenous wild type DRaf levels were essentially equivalent to those of wild type embryos with only one (1/245) lacking posterior Filzkörper (line L1, Figure II-1B, E). However, when DRaf$^{\Delta N114}$ was expressed at low maternal levels (~ 1/4 of endogenous DRaf level; 2 independent transgenic lines #a and #b; Figure II-1B), ~4.5% of the embryos assayed lacked posterior Filzkörper (Figure II-1D iii, E).

At such a reduced expression level (2 independent lines #a and #b, Figure II-1B), FL DRaf showed rescue of posterior pattern with Filzkörper development observed for a higher percentage of embryos (~98.5%, χ^2=9.91976318, P<0.01; Figure II-1D, E). In agreement with the cuticle phenotype, an abnormal posterior *tll* expression pattern (<13% EL) was observed more often for embryos that inherited truncated DRaf$^{\Delta N114}$ (78.8%, n=52) rather than full-length DRaf proteins (91%, n=78; χ^2=3.9386844, P<0.05, Figure II-1D), suggesting that DRaf$^{\Delta N114}$ was less active than FL DRaf in Torso RTK signaling.

To test our protein quantification assay, a more rigorous examination was conducted using western blot analysis. Three samples representing lystes of 6, 12, and 18 eggs from each line (*Draf$^{-/-}$; DRaf$^{\Delta N114}$*#a and *Draf$^{-/-}$; FL DRaf* #a) were loaded on to a SDS-PAGE gel. As shown in Figure II-2A, the intensity of DRaf and corresponding tubulin bands exhibits a roughly linear correlation with the number of eggs lysed (Figure II-2B). In addition, the normalized DRaf protein level was consistent among the three samples loaded for the same transgenic line (Figure II-2C), suggesting our western blots analysis was reliable. Importantly, we also addressed the question of maternal DRaf protein stability and whether deletion of the N-terminal region altered DRaf accumulation levels during Torso signal transduction. Embryonic lysates from eggs collected at 0-1, 1-2 and 2-3 hours after deposition were prepared (*Draf$^{-/-}$; DRaf$^{\Delta N114}$*#a and *Draf$^{-/-}$; FL DRaf* #a). As shown in Figure II-2D and E, DRaf protein levels remained roughly constant, indicating both FL DRaf and DRaf$^{\Delta N114}$ proteins are stable throughout the 0-3 hour period when the Torso pathway is active.

Next, we genetically altered the Torso pathway to create a sensitized signaling environment and compared the potential of DRaf$^{\Delta N114}$ and FL Raf proteins in this background. *tor*^{RL3} is a temperature-sensitive, recessive, gain-of-function allele of the Torso receptor. At the non-permissive temperature 25-29°, *tor*^{RL3}/*tor*^{RL3} mothers produce embryos that show broad *tll* expression at both anterior and posterior ends. These embryos develop and show deletion of central abdominal segments, and do not hatch (STRECKER *et al*. 1989). Eggs derived from females heterozygous for *tor*^{RL3} (*tor*^{RL3}/+) can hatch as larvae, however, some of these larvae show a gain-of-function phenotype with deletion of an abdominal segment(s). At 29°, we found 7.4% (n=553, Figure II-3D) of the larvae from *tor*^{RL3}/+ mothers showed deletion, fusion or broken abdominal denticle bands. In this genetic background, when expressed at comparable protein levels (Figure II-3A, B), FL DRaf enhanced the *tor*^{RL3} phenotype much more significantly than DRaf$^{\Delta N114}$, resulting in a greater number of embryos with central abdominal defects. We found 31.8% (n=422) embryos with FL DRaf proteins showed the gain-of-function phenotype (Figure II-3C, D), while only 17.1% (n=450) of the DRaf$^{\Delta N114}$ embryos showed such defects (Figure II-3C, D). We repeated these experiments using two additional, independently derived transgenic *FL DRaf* and *DRaf* $^{\Delta N114}$ lines and observed similar results (χ^2=51.063876, P<0.001; Figure II-3D).

To test if the cuticle phenotypes observed were due to alterations in the embryonic fate map, we determined the mRNA accumulation pattern for the *engrailed* (*en*) segmentation gene in ~ stage 11 embryos. The *en* mRNA wild type pattern is dependent on normal signaling in the Torso pathway. We found 36.1% (n=169) of the embryos from *tor*^{RL3}*/+; FL DRaf* mothers had at least one deleted, fused or broken *en* central abdominal stripe(s) (Figure II-3E iv). The segmentation defects observed were most likely due to the expansion of head

and/or tail domains and indicative of the gain-of-function phenotype. In contrast, only 33 of 141 (23.4%) embryos from *tor*^{RL3}/+; *DRaf*$^{\Delta N114}$ mothers had such defects (χ^2=6.38030206, P<0.02). Consistent with the cuticular phenotypes and *en* expression patterns, expansion in the domain of *tll* expression was observed more often for embryos from *tor*^{RL3}/+; *FL DRaf* mothers (32.0%, n=50) compared with those from *tor*^{RL3}/+; *Draf*$^{\Delta N114}$ females (13.1%, n=76; χ^2=5.50220096, P<0.02, Figure II-3E iii', iv'). Together, these *in vivo* studies consistently indicated that deletion of the N-terminus reduces the ability of DRaf to enhance the ectopic gain-of-function effects of *tor*^{RL3} and that these N-terminal residues could participate in Torso RTK signaling.

The contribution of DRaf's N-terminus to signaling appears to be dependent on the activity of the Torso receptor: Embryos lacking normal maternal Trunk (Trk) activity show little or no posterior *tll* expression (occasionally, trace-level *tll* expression was detected in 3-4 cells at the posterior embryo tip) and exhibit an abnormal expression pattern for the *engrailed (en)* segmentation gene. Instead of 3 thoracic and 9 abdominal *en* stripes as observed for embryos from wild type mothers (Figure II-4C i'), these Trk-deficient embryos have 3 thoracic and only 6-7 abdominal *en* stripes (Figure II-4C ii'), and exhibit terminal defects with deletion of all posterior structures (A8 denticle belt and Filzkörper; Figure II-4C ii). Interestingly, overexpression of FL DRaf partially restores the A8 denticle belt structure in embryos from *trunk*^{1}/*trunk*^{1} (*trk* $^{-}$/$^{-}$; lacks the last 16 amino acids) mothers (Figure II-4C iv). This result is consistent with our unpublished findings (K. H. BAEK and L. AMBROSIO) using the *trk*^{3} allele (encodes the first 89 amino acids) and a different method. Rescue of posterior structures for some Trk-deficient embryos was found after injection of wild type *DRaf* mRNA, also suggesting that accumulation of exogenous DRaf proteins promotes signaling in

this *trk*$^{-}$ background. However, expression of the *DRaf*$^{\Delta N114}$ transgene at a similar level failed to rescue the A8 denticle band defect in embryos from *trk* $^{-/-}$ mothers (Figure II-4 A, C iii). We repeated these experiments using two additional *FL DRaf* and *DRaf*$^{\Delta N114}$ transgenic lines and observed similar results (χ^2=82.8574882, P<0.001; Figure II-4D). Thus, FL DRaf appears to possess greater activity compared with DRaf$^{\Delta N114}$. To test if these cuticle phenotypes were correlated with segmentation gene expression, we examined the accumulation of *en* mRNA in early gastrulating embryos. All embryos from *trk*$^{-/-}$*; DRaf*$^{\Delta N114}$*/DRaf*$^{\Delta N114}$ mothers had 3 thoracic and only 6-7 abdominal *en* stripes (Figure II-4C iii'), while some embryos (12.5%, n=24) derived from *trk*$^{-/-}$*; FL DRaf/FL DRaf* females had 3 thoracic and 8 abdominal *en* stripes (χ^2=3.97058824, P<0.05; Figure II-4C iv'). This indicated that addition of FL DRaf but not DRaf$^{\Delta N114}$ proteins partially restored posterior Torso RTK signaling in the *trk*$^{-}$ background. Consistent with this hypothesis, as shown in Figure II-4C iii'' and iv'', partial rescue of posterior *tll* mRNA expression was detected in some cellular blastoderm embryos derived from *trk*$^{-/-}$*; FL DRaf/FL DRaf* females (8.1%, n=37) but not for those derived from *trk* $^{-/-}$*; DRaf* $^{\Delta N114}$*/DRaf* $^{\Delta N114}$ mothers (n=52, χ^2=4.36329353, P<0.05). Together, these data consistently suggest that the absence of the N-terminal segment reduces the signaling potential of DRaf and the N-terminus can contribute to Torso RTK terminal signaling in a positive manner.

Sprenger *et al.* (1993) previously observed a low level of Torso receptor phosphorylation in eggs derived from *trk* loss-of-function, but not *tor* loss-of-function females. Therefore, a small amount of Torso signal activity may exist in our *trk* $^{-}$ background. This may be due to 1.) the presence of active Torso-like (Tsl) ligand, 2.)potential residual Trunk activity, considering the molecular lesion of the *trk*^{1} allele we used (lacks only the last

16 amino acids), or 3.) the intrinsic activity of the Torso receptor. This activity could allow rescue of posterior structures by *FL DRaf* expression. If so, the contribution of the N-terminus to terminal signaling is likely sensitive to such upstream events. Thus, we examined the consequences of DRaf expression in embryos from tor^{XR1}/tor^{XR1} ($tor^{-}/^{-}$; protein null) mothers that lacked the Torso receptor. We found that expression of FL DRaf or $DRaf^{\Delta N114}$ failed to restore terminal structures for these embryos (Table II-1). This indicates the contribution of DRaf's N-terminal residues to signaling is dependent on activity of the receptor.

The N-terminus of DRaf contains a novel conserved region and has a high content of helical secondary structure: We analyzed the amino acid sequence of DRaf's N-terminus using several bioinformatics tools, to obtain hints regarding its structure, and perhaps mechanism(s) of its functional role(s). A PROSITE motif search showed a putative protein kinase C (PKC) phosphorylation site within the "T-S-K" motif of the N-terminus (positions 60-62; SIGRIST *et al.* 2002). Phosphorylation site prediction by NetPhos 2.0 suggested that the Thr in this "T-S-K" motif had a high phosphorylation potential (Figure II-5A; BLOM *et al.* 1999). Predictions of secondary structure for the N-terminal region using GORV, PHD and Predator indicated a high α-helical propensity (Figure II-5A; FRISHMAN and ARGOS 1996; ROST *et al*., 1994; GARNIER *et al.*, 1996; COMBET *et al.* 2000).

A blastp search of other organisms with DRaf's N-terminal sequence identified honeybee Raf, chick C-Rmil, and BRaf proteins of sea urchin, zebrafish, frog and human. The region containing amino acid 19 to 77 of DRaf showed homology between candidates. These sequences were aligned using ClustalW, and are shown in Figure II-5A (COMBET *et al.* 2000). Overall, the amino acids showed 18.6% identity and 47.5% similarity, and we term

this region CRN, Conserved Region N-terminal. Interesting features of CRN include the putative phosphorylation site, and a propensity to form two α-helical structures. This suggests that the N-terminal region of DRaf may have function(s) shared by other BRaf proteins.

The conserved structural features, including α-helical propensity, may be related to the functional role(s)/regulatory mechanism(s) of DRaf's N-terminus. To confirm the prediction attained by bioinformactics tools, circular dichroism (CD) spectral measurement of the N-terminal part of DRaf (amino acid 1-117, $DRaf^{N117}$) was performed after its expression and purification *in vitro* (see MATERIALS and METHODS). As shown in Figure II-5B, a bilobed spectrum with local minima at ~209.4 nm and at ~221.4 nm was observed, indicating the relatively high content of helical secondary structure for DRaf's N-terminus. The estimated helix content of $DRaf^{N117}$ is ~77% based on the CD spectra data analysis using DICHROWEB (WHITMORE, *et al.*, 2008, http://dichroweb.cryst.bbk..ac.uk/html/home.shtml). This result bolsters the predictions by GORV, PHD and Predator.

The N-terminus assists in association of DRaf's RBD with small GTPases Ras1 and Rap1 *in vitro*: Fischer *et al.* (2007) found that association of BRaf with HRas was facilitated by N-terminal sequences, *in vitro*. To examine if the presence of DRaf's N-terminus can affect Ras1 binding, we tested interaction between Ras1ΔCAAX and DRaf's RBD (Ras binding domain) using the yeast two-hybrid assay. A stronger interaction with Ras1ΔCAAX was detected when N-terminal residues were linked to RBD in both solid-support (data not shown) and liquid quantitative ß-Galactosidase assays ($P<0.05$, *t test*; Figure II-6B), suggesting that the N-terminus may assist in association of DRaf with Ras1. This is consistent with results obtained for BRaf (FISCHER *et al.* 2007).

No direct interaction was detected between Ras1ΔCAAX and isolated N-terminal residues of DRaf (Figure II-6B, and solid-support data not shown). Thus, the N-terminus appears to contribute to Ras1 binding, but as an isolated protein fragment cannot directly interact with Ras1. Arg174 located in DRaf's RBD region is essential for its association with Ras1 and substitution of Arg174 to Leu in RBD (RBD^{R174L}) abolishes Ras1 binding (FABIAN *et al*. 1994; LI *et al*. 1998). We found that N-terminal residues cannot restore Ras1 interaction when linked with RBD^{R174L} (Figure II-6B, and solid-support data not shown). This indicated the effects of the N-terminus were dependent on interaction between RBD and Ras1.

Moreover, we tested the idea that the conserved CRN region (19-77) might be essential for the contribution of DRaf's N-terminus to Ras1ΔCAAX binding. Deletion of the first seventeen N-terminal amino acids (Δ17NRBD) did not change Ras1ΔCAAX binding. However, if N-terminal amino acids including CRN were removed (Δ77NRBD), interaction with Ras1ΔCAAX was reduced to a level similar to that observed by deletion of the entire N-terminus (amino acids 1-114; Figure II-6A, B). Together, these findings suggested the hypothesis that N-terminal residues of DRaf can assist in Ras1 interaction through a CRN-mediated mechanism(s).

The small GTPase Rap1, a close relative of Ras1, is known to interact with DRaf and play a role in Torso RTK signaling *in vivo* (MISHRA *et al*. 2005). To examine if DRaf's N-terminus affects its association with Rap1, we tested interaction between Rap1ΔCAAX and DRaf's RBD (Figure II-6C). A stronger interaction with Rap1ΔCAAX was detected when the N-terminus was linked to RBD, similar to our findings with Ras1 ($P<0.05$, *t test*). Furthermore, the conserved CRN region (19-77) seems essential for the contribution of the

N-terminus to this interaction with Rap1, suggesting a CRN-mediated mechanism(s) may be a general feature for its binding to both Ras1 or Rap1.

Discussion

In our study, a novel region (amino acids 19-77) within DRaf's N-terminus, conserved for Raf genes of most invertebrates and BRaf genes of vertebrates, was identified and termed Conserved Region N-terminal (CRN). This conserved region has not been described by others, but potential roles for the extended N-terminus have been proposed in two reports. Terai and Matsuda (2006) found that in Hela cells, the N-terminus of BRaf may mediate Raf dimerization to generate BRaf-BRaf or BRaf-CRaf complexes, and play an important regulatory role in calcium-induced BRaf activation. However, Fischer *et al.* (2007) reported that deletion of BRaf's N-terminus did not affect BRaf-CRaf dimer formation. Instead, they found that N-terminal residues appeared to facilitate interaction with HRas *in vitro*. In accordance with their data, stronger interactions between DRaf's RBD (Ras binding domain) and the small GTPase Ras1ΔCAAX were observed when N-terminal and RBD sequences were linked in our yeast two-hybrid analysis. This suggested that the N-terminus might assist in Ras1 binding. Furthermore, the identity of specific residues in the N-terminus that might participate in Ras1 binding were mapped to the CRN region (amino acid 19-77). Two known Raf motifs, RBD and CRD (cysteine rich domain), are involved in Raf's interaction with Ras. Our studies, and results obtained by Fischer *et al.* (2007) using BRaf, suggest that the N-terminal residues of DRaf and BRaf proteins, particularly the CRN region, might be another element that plays a role(s) in Ras-Raf coupling.

The small GTPase Rap shares with Ras nearly identical Raf binding regions that comprise switch 1 and the lipid moiety (HARIHARAN 2005). Rap functions as an antagonist of Ras in regulating CRaf activity (COOK *et al.* 1993), but can activate BRaf in a parallel way with Ras (OHTSUKA *et al.* 1996). Isoform-specific features of different Raf family members may explain their distinct responses to Rap. In flies, both Ras1 and Rap1 can interact with and activate DRaf (MISHRA *et al.* 2005). Thus, it was reasonable to test whether DRaf's N-terminus including CRN might also assist in Rap1 binding. In agreement with this idea, stronger interaction between RBD and Rap1ΔCAAX was observed when DRaf's CRN and RBD sequences were linked *in vitro*, further suggesting that the N-terminus may contribute to both Ras1 and Rap1 binding potentially through a CRN-mediated mechanism(s) *in vivo*.

What is the molecular mechanism(s): No direct interaction between Ras1 or Rap1 and the isolated DRaf N-terminal segment (amino acids 1-117) was detected, or when the N-terminus was linked with the Ras1/Rap1 binding-deficient RBD^{R174L}. Thus, the contribution of DRaf's N-terminal residues to Ras1 and Rap1 binding requires the presence of RBD. It is possible that the CRN-containing N-terminus may assist in Raf-Ras interaction by making RBD more accessible to Ras1 and/or in a sequential manner, subsequent to RBD-Ras1 interaction, by stabilizing the RBD-Ras1 complex. Deletion of CRN may result in conformational or structural changes that reduce Ras1 binding affinity. Structural analysis of these complexes may provide important clues and help to understand the molecular mechanism(s) by which CRN assists in Ras-Raf interaction. Our computational analysis suggested conserved CRN has the propensity to form two α-helical structures (α1 and α2; Figure II-5A) and contains a putative phosphorylation motif "T-S-K" located in α2. In agreement, DRaf's N-terminus (amino acids 1-117) was folded *in vitro* and had a high

content of helical secondary structure (Figure II-5B). These findings may help to establish a basis for future determination of molecular structure.

Although no verified binding partner(s) for DRaf or BRaf's N-terminus has been identified, it is still possible that CRN may interact with other regulatory factors *in vivo*, that may affect Ras or Rap binding and/or function in activation of DRaf and BRaf. If so, the conserved structural features of CRN most likely relate to these regulatory events *in vivo*. Site-directed mutagenesis of conserved sites/motifs could provide useful information regarding the molecular mechanism(s) of CRN's role in the activation of DRaf and BRaf.

Torso RTK signal is differentially elevated by overexpression of FL DRaf and DRaf$^{\Delta N114}$ *in vivo*: We initiated our *in vitro* studies of DRaf's N-terminus based on our *in vivo* findings using both loss- and gain-of-function genetic assays that deletion of N-terminal residues consistently reduces DRaf's signal potential in the Torso pathway. When expressed at high levels, FL DRaf enhanced the gain-of-function effects of the *tor^{RL3}* allele much more significantly than DRaf$^{\Delta N114}$. In embryos from *trk* $^{-/-}$ mothers, addition of FL DRaf, but not DRAF$^{\Delta N114}$, partially restored the A8 denticle belt structure (Figure II-4). These findings indicate that the N-terminus can play a positive role(s) in Torso RTK signaling. Interestingly, the contribution of DRaf's N-terminus in the Torso pathway appeared to be dependent on upstream receptor activity, suggesting its role in transmission of the signal. Together with our yeast two-hybrid data, as well as the results obtained by Fischer *et al.* (2007) for BRaf, we propose that the presence of N-terminal residues may facilitate the association of DRaf with the upstream regulators Ras1 and Rap1, thereby assisting in transmission of the RTK signal *in vivo*.

For instance, in the *trk*$^{-}$ background, a small amount of active GTP-Ras1 and GTP-Rap1 are likely present, mostly due to activation by residual upstream Trunk activity, the presence of Torso-like ligand, and/or the intrinsic activity of the Torso receptor. The *trk*^{1} mutation used in this analysis results in protein truncation at the last 16 amino acids. It is possible that over-expression of FL DRaf proteins in this background increases the likelihood of interaction between abundant DRaf proteins and membrane bound GTP-Ras1 or GTP-Rap1. This in turn, could elevate RTK signal and partially restore development of A8 posterior structures in some embryos. On the other hand, deletion of the N-terminus could destabilize Ras1-DRaf (or Rap1-DRaf) coupling or decrease the duration of interaction, resulting in reduced DRaf signal transmission. This may explain why expression of DRaf$^{\Delta N114}$ failed to rescue the A8 denticle belt in embryos from *trk*$^{-/-}$ mothers.

Why are only minor differences detected *in vivo* between FL DRaf and DRaf$^{\Delta N114}$: Previously, an auto-inhibitory role had been assigned to residues compromising the first half of the DRaf protein, in addition to their functions in promoting its activity. Deletion of the N-terminal 1-272 (including the N-terminus and CR1) or 1-402 (including the N-terminus, CR1 and CR2) of DRaf at least partially relieved these negative effects (BAEK *et al.* 1996). Here, although removal of the N-terminal 1-114 residues did not result in constitutive DRaf$^{\Delta N114}$ activity in embryos lacking the maternal Torso receptor (Table II-1), it is still possible that the N-terminus may contribute to auto-inhibitory effects. Together with CR1 and CR2, these N-terminal residues (1-114) may help maintain DRaf's inactive conformation. If so, the N-terminus might play dual roles, both positively and negatively regulating DRaf. Therefore, its contribution to signaling may be neutralized by this auto-inhibition and consequently result in a subtle *in vivo* effect. If so, selective mutagenesis of the "inhibitory" motifs/sites in the N-

terminal region or removal of other co-factors involved in its negative regulation may amplify signaling differences between FL DRaf and DRaf$^{\Delta N114}$.

Ras binding has been thought crucial to recruit Raf to the plasma membrane and promote its RTK signaling activity. However, the Drosophila Torso pathway appears tolerant of alterations in Ras1-DRaf coupling (HOU *et al*. 1995). Draf ^{C110} has a R174L point mutation in the RBD domain and likely comprised for Ras1 binding (LI *et al*. 1998). The RBD^{R174L} is Ras binding-deficient in our yeast two-hybrid assay (Figure II-6B). However, *tll* expression patterns and cuticles of the embryos derived from mothers with *Draf^{C110}/Draf^{C110}* germ cells were indistinguishable from those of wild type embryos (MELNICK *et al*. 1993), suggesting a mechanism(s) independent of RBD-Ras1 interaction might function in recruiting DRaf to the plasma membrane. In agreement with this model, Rizzo *et al*. (2000) found membrane translocation of CRaf could be mediated by its interaction with phosphatidic acid (PA), and independent of Ras binding. This PA binding site is also conserved in ARaf, BRaf, and DRaf. Thus, Draf^{C110} could be recruited to the cell surface by associating with PA. Moreover, it is known that Raf's CRD participates in Ras binding through its interaction with the lipid moiety of Ras (WILLIAMS *et al.* 2000; THAPAR *et al.* 2004). Once at the membrane, it is also possible that the interaction between Draf^{C110}'s CRD and Ras1 could further promote its membrane attachment and result in relatively normal Torso signal production. In this study, the presence of RBD, CRD and the potential PA binding site may be sufficient to promote DRaf's activation in Torso signaling. This may explain why at ~ endogenous wild type protein level maternally expressed DRaf$^{\Delta N114}$ is able to rescue the embryonic terminal defects of *Draf$^{11-29}$* mutants (Figure II-1B, C and E). Together, considering the Torso pathway's tolerance of alterations in Ras1-DRaf coupling

and the minor role DRaf's N-terminus plays in Ras1 binding, it is reasonable that the phenotypic consequences of removing these N-terminal residues ($DRaf^{\Delta N114}$) are not great in Torso signaling.

The subtle phenotypic effects of DRaf's N-terminus could also be due to compensation provided by potential autoregulatory feedback or alternative redundant processes in the *in vivo* system. In our study, the expression of DRaf proteins at a low level (~1/4 endogenous wild type level) appeared to sensitize the assay system. We found deletion of the N-terminus seemed to increase the threshold of DRaf protein levels required for normal signaling. Furthermore, by adding one copy of the ectopic tor^{RL3} allele or removing wild type maternal Trunk activity we apparently increased the sensitivity of the Torso pathway. These allowed the embryonic terminal system to display enhanced differences between FL DRaf and $DRaf^{\Delta N114}$ proteins.

The biological implications of the N-terminal region: Why is this N-terminus with its "subtle" functional effects conserved during evolution, and what is its biological relevance? There are numerous RTK pathways functioning in Drosophila cellular and developmental processes. In spite of the identical Ras-Raf-MEK signal cassette they share, these RTK pathways can lead to different biological responses. Previous studies indicated that such specificity might be due to the difference in the intensity and/or duration of the signal (GHIGLIONE *et al.* 1999; KERKHOFF and RAPP 1998; WOODS *et al*. 1997; WOODS *et al.* 2001). This suggested that the magnitude of Raf signal could function as a critical determinant of biological responses. Participation of multiple DRaf elements in Ras1 or Rap1 binding could be a good strategy to modulate its activity. Normally, tight association with Ras1 or Rap1 through RBD and CRD regions is required and sufficient to initiate the

activation of DRaf, while minor adjustments/regulation of interaction by the CRN region could optimize signaling potential and reduce variability. Thus, the extended N-terminus including CRN may play a role(s) as one element in a multi-domain effort to promote DRaf's interaction with Ras1 and Rap1, participating and assisting in regulation to reliably attain maximal signal output.

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Tables

TABLE 1. Expression of FL DRaf or DRaf$^{\Delta N114}$ did not result in rescue of the A8 dentical belt in embryos produced by *tor^{XR1}/tor^{XR1}* females

	#(%) embryos whose most posterior structure belongs to		
Maternal genotype	A6/A7 segment	A8 denticle belt	Total#
tor^{XR1}/tor^{XR1}	117 (100)	0 (0)	117
tor^{XR1}/tor^{XR1}; DRaf$^{\Delta N114}$ #1/DRaf$^{\Delta N114}$ #3	72 (100)	0 (0)	72
tor^{XR1}/tor^{XR1}; DRaf$^{\Delta N114}$ #2/DRaf$^{\Delta N114}$ #2	77 (100)	0 (0)	77
tor^{XR1}/tor^{XR1}; DRaf$^{\Delta N114}$ #3/DRaf$^{\Delta N114}$ #3	98 (100)	0 (0)	98
tor^{XR1}/tor^{XR1}; FL Draf #1/FL DRaf #1	61 (100)	0 (0)	61
tor^{XR1}/tor^{XR1}; FL Draf #2/FL DRaf #2	70 (100)	0 (0)	70
tor^{XR1}/tor^{XR1}; FL Draf #3/FL DRaf #3	81 (100)	0 (0)	81

Figure Legends

Figure II-1. Rescue of posterior structures in embryos derived from *Draf*$^{-/-}$ female germ cells by expression of full-length *DRaf* or truncated *DRaf*$^{\Delta N114}$ transgenes. (A) Schematic representations of full-length DRaf (FL DRaf) with 739 amino acids and truncated DRaf$^{\Delta N114}$ proteins. In addition to the 3 conserved regions (CR1, CR2 and CR3), FL DRaf has an extended N-terminus. (B) Western analysis of embryonic DRaf proteins from eggs produced by *Draf* $^{11-29}$/*Draf* $^{11-29}$ (*Draf* $^{-/-}$), wild type (WT), *Draf* $^{-/-}$; *DRaf* $^{\Delta N114}$ (3 independent lines, L1 with ~1X endogenous DRaf level, #a and #b with ~1/4 endogenous DRaf level) and *Draf* $^{-/-}$; *FL DRaf* (2 independent lines, #a and #b with ~1/4 endogenous DRaf level) germline clone bearing females. Lysate was prepared from eggs at 0-3 hours after egg deposition. Full-length DRaf (~90 KD) and DRaf$^{\Delta N114}$ (~77 KD) proteins are denoted by arrows. Lysate of eggs from *Draf* $^{-/-}$ germ cells was used as a negative control. α-tubulin (α-tub) was used as the loading control. (C) A bar graph representing relative levels of DRaf proteins normalized with α-tubulin. (D) Cuticles of mature embryos derived from wild type (WT), *Draf* $^{-/-}$, *Draf* $^{-/-}$; *DRaf* $^{\Delta N114}$ and *Draf* $^{-/-}$; *FL DRaf* female germ cells are shown in left panels. Accumulation of *tll* mRNA in cellular blastoderm embryos was detected by *in situ* hybridization (Right panels). Posterior *tll* expression is solely dependent on the Torso pathway and used as a marker for Torso RTK signaling. Anterior expression of *tll* is regulated by another pathway(s) in addition to Torso signaling, is more complex, and is used as an internal control for staining here. Wild type embryos show (i) normal Filzkörper structure (arrow), (i') *tll* mRNA accumulation at the posterior (approximately 0-15% embryo length, EL), and an anterior head "stripe" (~75-85% EL). Embryos derived from *Draf* $^{-/-}$

germ cells lack (ii) posterior structures (A8 denticle belt, Filzkörper) and (ii') posterior *tll* mRNA expression. (iii) An embryonic cuticle derived from *Draf*$^{-/-}$*; DRaf*$^{\Delta N114}$ germ cell lacks the Filzkörper structure. (iii') A reduced posterior *tll* expression domain is at ~0-8% EL in an embryo from *Draf*$^{-/-}$*; DRaf*$^{\Delta N114}$ germline clone bearing mother. (iv) Filzkörper structure (arrow) and (iv') normal expression pattern of *tll* mRNAs are rescued by FL DRaf expression for embryos derived from *Draf*$^{-/-}$*; FL DRaf* maternal germ cells. (E) A bar graph showing the percentage of embryos without Filzkörper (Fk) structures. When expressed at low maternal level (~1/4 endogenous level), embryos without Fk were found more often for those that inherited truncate Draf$^{\Delta N114}$ rather than full length DRaf proteins (χ^2=9.91976318, P<0.01).

Figure II-2. Verification of DRaf protein quantitation assays and stability of DRaf proteins in early embryos. (A) Three samples representing lystes of 6, 12, and 18 eggs for each line (*Draf*$^{-/-}$*; DRaf*$^{\Delta N114}$#a and *Draf*$^{-/-}$*; FL DRaf* #a) were loaded for western blot analysis. Full-length DRaf (~90 KD) and DRaf$^{\Delta N114}$ (~77 KD) proteins are denoted by arrows. Lysate of eggs from *Draf*$^{11-29}$*/Draf*$^{11-29}$ (*Draf*$^{-/-}$)germ cells was used as a negative control. α-tubulin levels were probed as a loading control. (B) Bar graph showing relative intensity of DRaf (dark bar) and α-tubulin (gray bar) bands. (C) A bar graph depicting normalized DRaf protein level from (A). (D) Western analysis of embryonic DRaf proteins from eggs collected at 0-1, 1-2 and 2-3 hours after deposition and produced by *Draf*$^{-/-}$, *Draf*$^{-/-}$*; DRaf*$^{\Delta N114}$ (line #a) and *Draf*$^{-/-}$*; FL DRaf* (line #a) germline-bearing females. Full-length DRaf (~90 KD) and DRaf$^{\Delta N114}$ (~77 KD) proteins are denoted by arrows. α-tubulin was used as the loading control. (E) Normalized DRaf protein level from (D) is shown in this bar graph depicting the stable accumulation of these DRaf proteins.

Figure II-3. Gain-of-function effects of *tor*^{RL3} are differentially enhanced by expression of *FL DRaf* and *DRaf*$^{\Delta N114}$ transgenes. (A) Western analysis of embryonic DRaf proteins from eggs (0-3 hours) produced by *tor*^{RL3}/+, *tor*^{RL3}/+; *DRaf* $^{\Delta N114}$ (3 independent lines, #1, #2, #3), or *tor*^{RL3}/+;*FL DRaf* (3 independent lines, #1, #2, #3) females at 29°. Full-length DRaf (~90 KD) and DRaf$^{\Delta N114}$ (~77 KD) proteins are denoted by arrows. α-tubulin was used as the loading control. (B) Normalized DRaf protein level from (A) is shown as a bar graph. (C) Cuticles of mature embryos are shown. (i) A wild type (WT) embryo exhibits normal cuticle pattern with 8 abdominal denticle belts. (ii) An embryonic cuticle derived from *tor*^{RL3}/+; *FL DRaf* mother has one broken abdominal denticle band (arrow head), and is missing one central abdominal denticle belt (arrow). (D) Percent of embryonic cuticles with gain-of-function phenotypes is shown in this bar graph. Gain-of-function effects of *tor*^{RL3} were differentially enhanced by FL DRaf and DRaf$^{\Delta N114}$ proteins (χ^2=51.063837, P<0.001). (E) Expression of *engrailed* (*en*) at ~stage 11 (Left panels) and accumulation of *tailless* (*tll*) mRNA at cellular blastoderm stage (Right panels) in embryos from WT, *tor*^{RL3}/+, *tor*^{RL3}/+;*DRaf*$^{\Delta N114}$, or *tor*^{RL3}/+;*FL DRaf* mothers: Examples of embryos derived from (i) WT, (ii) *tor*^{RL3}/+, and (iii) *tor*^{RL3}/+;*DRaf*$^{\Delta N114}$ mothers exhibit normal *en* mRNA pattern with 3 thoracic (T1-3) and 9 abdominal (A1-9) expression stripes. (iv) An embryo from *tor*^{RL3}/+; *FL DRaf* mother with partial deletion of *en* stripes (arrow) in a region that gives rise to central abdominal segmental pattern is shown. Examples of embryos derived from (i') WT and (ii) *tor*^{RL3}/+ mothers exhibiting a normal *tll* mRNA pattern. (iii') An embryo from a *tor*^{RL3}/+; *DRaf* $^{\Delta N114}$ mother shows slightly expanded posterior expression domain of *tll*. (iv') An embryo derived from *tor*^{RL3}/+;*FL DRaf* females exhibits expanded domain of *tll* expression for both anterior and posterior regions.

Figure II-4. Effects of FL DRaf and DRaf$^{\Delta N114}$ expression on posterior development in embryos derived from *trk^{1}/trk^{1}* mothers. (A) Western analysis of embryonic DRaf proteins from eggs (0-3 hours) produced by *Draf$^{11-29}$/Draf$^{11-29}$* (*Draf$^{-/-}$*), wild type (WT), *trk^{1}/trk^{1}* (*trk$^{-/-}$*), *trk$^{-/-}$; DRaf$^{\Delta N114}$/DRaf$^{\Delta N114}$* (3 lines, #1/#3, #2/#2, #3/#3) and *trk$^{-/-}$; FL DRaf/FL DRaf* (3 lines, #1/#1, #2/#2, #3/#3) females. Full-length DRaf (~90 KD) and DRaf$^{\Delta N114}$ (~77 KD) proteins are denoted by arrows. Embryonic lysate from *Draf$^{-/-}$* germline clone females was used as a negative control. α-tubulin was used as the loading control. (B) Normalized DRaf protein level from (A) is shown in the bar graph. (C) Representative cuticles of mature embryos derived from wild type (WT), *trk$^{-/-}$*, *trk$^{-/-}$; DRaf$^{\Delta N114}$/DRaf$^{\Delta N114}$*, or *trk$^{-/-}$; FL DRaf/FL DRaf* females are shown (Left panels). Accumulation of *en* (Middle panels) and *tll* (Right panels) mRNAs were detected by *in situ* hybridization. (i) A wild type (WT) embryo has normal cuticle pattern with 8 abdominal denticle belts and Filzkörper structure. (i') A WT embryo at gastrula stage has 3 thoracic (T1-3) and 9 abdominal (A1-9) *en* stripes. (i'') A WT embryo at cellular blastoderm stage exhibits a normal posterior expression domain of *tll*. (ii) Cuticle of a mature embryo from a *trk$^{-/-}$* mother is missing posterior structures (A8 segment, Filzkörper). (ii') A gastrulae embryo from a *trk$^{-/-}$* mother has abnormal *en* expression pattern with only 7 abdominal stripes. (ii'') A cellular blastoderm embryo from a *trk$^{-/-}$* mother lacks posterior *tll* expression. (iii) An embryonic cuticle from a *trk$^{-/-}$*; *DRaf$^{\Delta N114}$/DRaf$^{\Delta N114}$* mother lacks posterior structures (A8 denticle belt, Filzkörper). (ii') A gastrula embryo from a *trk$^{-/-}$*; *DRaf$^{\Delta N114}$/DRaf$^{\Delta N114}$* mother has only 7 abdominal *en* stripes. (ii'') A cellular blastoderm embryo from a *trk$^{-/-}$*; *DRaf$^{\Delta N114}$/DRaf$^{\Delta N114}$* mother lacks posterior expression of *tll* mRNA. Expression of FL DRaf (iv) restores the A8 denticle belt (arrow), (iv') partially rescues the *en* mRNA pattern (8 abdominal stripes), and (iv'')

posterior *tll* expression (arrow) in embryos lacking maternal Trk activity. (D) Effect of *FL DRaf* or *DRaf*$^{\Delta N114}$ transgene expression on A8 denticle development in embryos derived from *trk*$^{-/-}$ mothers (percent of embryonic cuticles with A8 denticle belt). Shown are results using transgenic *DRaf* $^{\Delta N114}$ or *FL DRaf* lines that express DRaf protein at similar levels. Expression of exogenous FL DRaf, but not DRaf$^{\Delta N114}$, results in partial rescue of A8 denticle belt in some embryos derived from *trk* $^{-/-}$ mothers (χ^2=82.8574882, P<0.001).

Figure II-5. The N-terminus of DRaf contains a novel conserved region and has a high content of helical secondary structure. (A) Drosophila Raf (NP_525047; 739 amino acids) has in addition to its three conserved regions (CR1-3), an extensive N-terminus. A novel region (amino acids 19-77) within the N-terminus is conserved in honeybee Raf (*A. mellifera* XP_396892); frog BRaf (*X. laevis* AAU29410); chicken C-Rmil (*G. gallus* CAA47436); human BRaf (*H. sapiens* NP_004324); zebrafish BRaf (*D. rerio* BAD16728), sea urchin BRaf (*S. purpuratus* XP_781094), and termed Conserved Region N-terminal (CRN). Sequences of CRN were aligned using ClustalW (identities were denoted as "*****", strong and weak similarities were denoted as "**:**" and "**.**", respectively in consensus line, http://www.ebi.ac.uk/tools/clustalw/), and the conserved residues were shaded using BOXSHADE (identities in black, similarities in gray, http://www.ch.embnet.org/software/box.form.html). Secondary structure prediction with GORV indicates CRN has the propensity to form two α-helices (α1 and α2). The putative PKC phosphorylation site DRaf's Thr60 is framed. (B) Circular dichroism (CD) spectral measurement of DRaf's N-terminus (amino acids 1-117) *in vitro*: The bilobed spectrum (arrows, local minima at ~209.4 nm and at ~221.4 nm) indicative of helical secondary structure is shown.

Figure II-6. Effects of the extended N-terminus of DRaf on Ras1 and Rap1 binding. (A) Schematic representations of different DRaf constructs used for yeast two-hybrid analysis. (B) Interactions between DRaf's RBDs and Ras1ΔCAAX: Removal of CRN or the entire N-terminal region reduces Ras1ΔCAAX binding ($P<0.05$, *t test*). (C) Interactions between DRaf's RBDs and Rap1ΔCAAX: Removal of CRN or the entire N-terminal region reduces Rap1ΔCAAX binding ($P<0.05$, *t test*).

Figure II-1

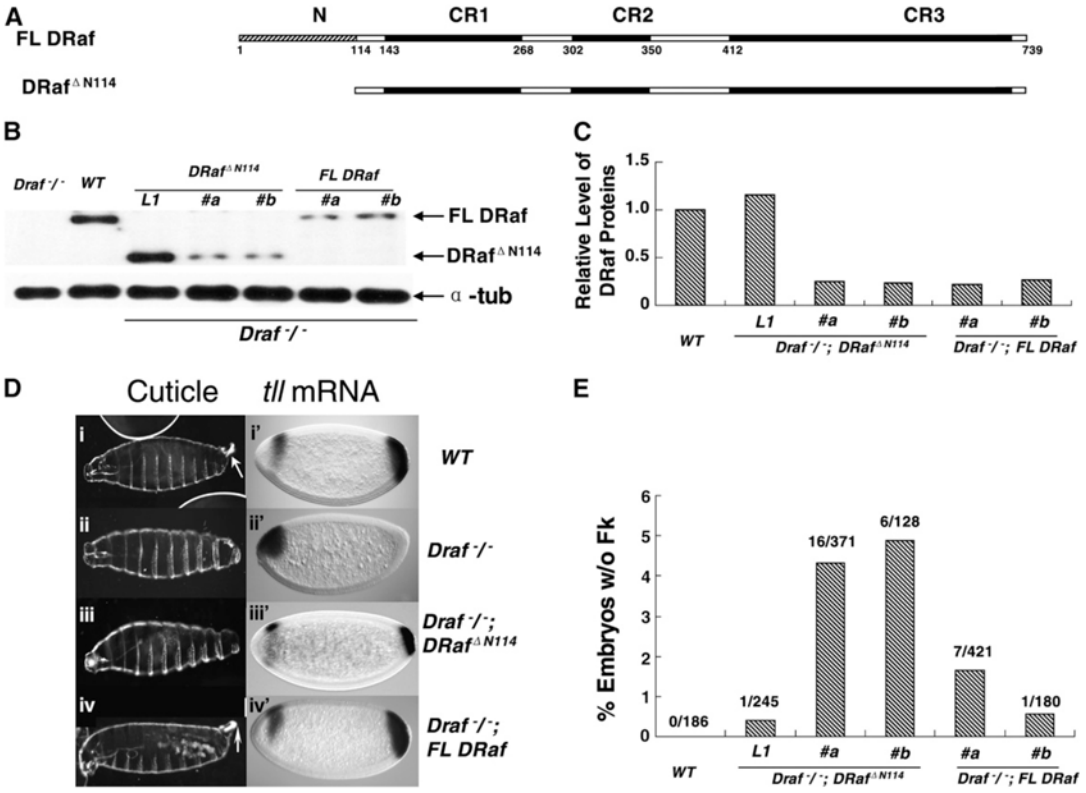

A
FL DRaf
N
CR1
CR2
CR3
1
114
143
268
302
350
412
739
DRaf ΔN114
B
Draf -/-
WT
DRaf ΔN114
FL DRaf
L1
#a
#b
#a
#b
FL DRaf
DRaf ΔN114
α -tub
Draf -/-
C
Relative Level of DRaf Proteins
1.5
1.0
0.5
0
WT
L1
#a
#b
#a
#b
Draf -/-; DRaf ΔN114
Draf -/-; FL DRaf
D
Cuticle
tll mRNA
i
i'
WT
ii
ii'
Draf -/-
iii
iii'
Draf -/-; DRaf ΔN114
iv
iv'
Draf -/-; FL DRaf
E
% Embryos w/o Fk
6
5
4
3
2
1
0
0/186
1/245
16/371
6/128
7/421
1/180
WT
L1
#a
#b
#a
#b
Draf -/-; DRaf ΔN114
Draf -/-; FL DRaf

Figure II-2

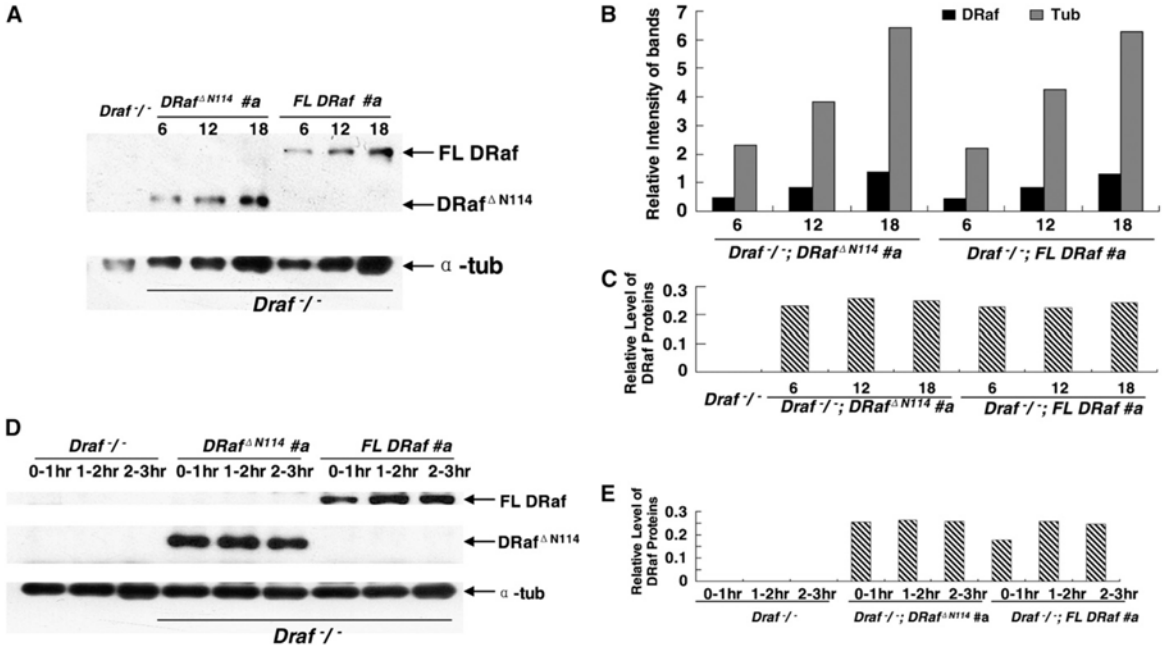

A
Draf -/-
DRaf ΔN114 #a
FL DRaf #a
6 12 18 6 12 18
FL DRaf
DRaf ΔN114
α -tub
Draf -/-
B
Relative Intensity of bands
DRaf
Tub
Draf -/-; DRaf ΔN114 #a
Draf -/-; FL DRaf #a
C
Relative Level of DRaf Proteins
D
0-1hr 1-2hr 2-3hr
E

Figure II-3

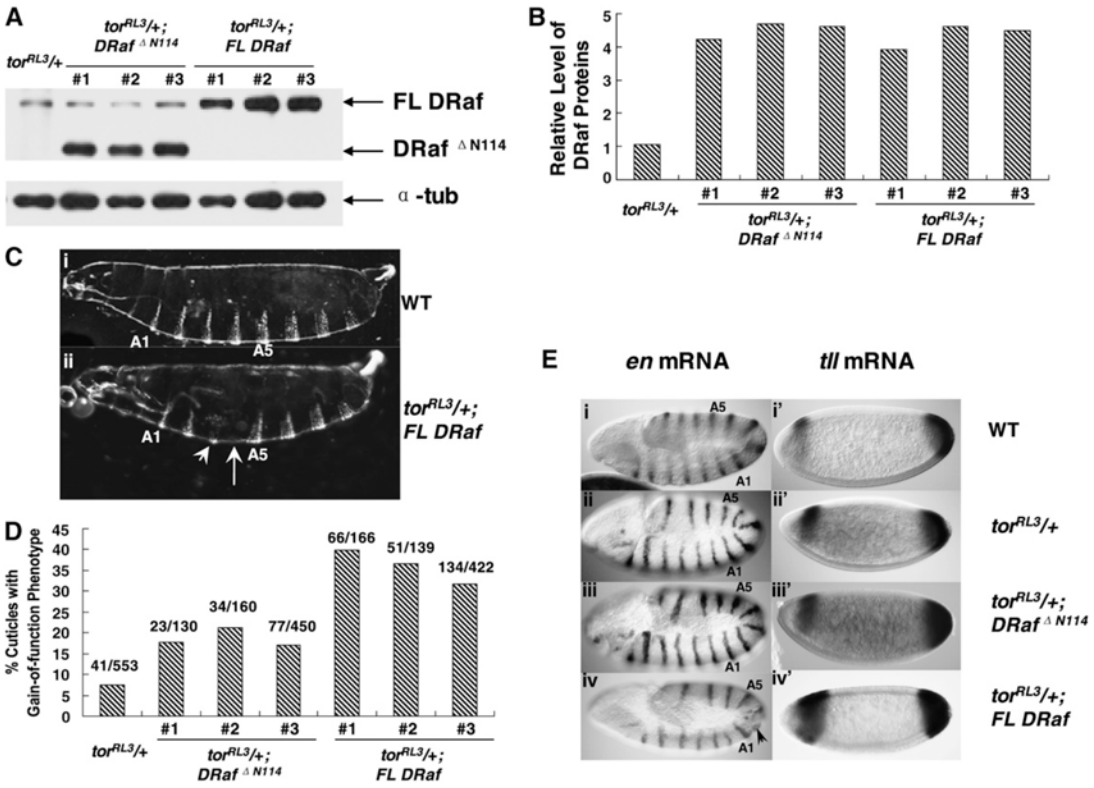

A
tor RL3/+
tor RL3/+; DRaf ΔN114
tor RL3/+; FL DRaf
#1 #2 #3 #1 #2 #3
FL DRaf
DRaf ΔN114
α-tub
B
Relative Level of DRaf Proteins
0 1 2 3 4 5
tor RL3/+
tor RL3/+; DRaf ΔN114
tor RL3/+; FL DRaf
C
i
WT
A1
A5
ii
tor RL3/+; FL DRaf
D
% Cuticles with Gain-of-function Phenotype
0 5 10 15 20 25 30 35 40 45
41/553
23/130
34/160
77/450
66/166
51/139
134/422
E
en mRNA
tll mRNA
WT
tor RL3/+
tor RL3/+; DRaf ΔN114
tor RL3/+; FL DRaf

Figure II-4

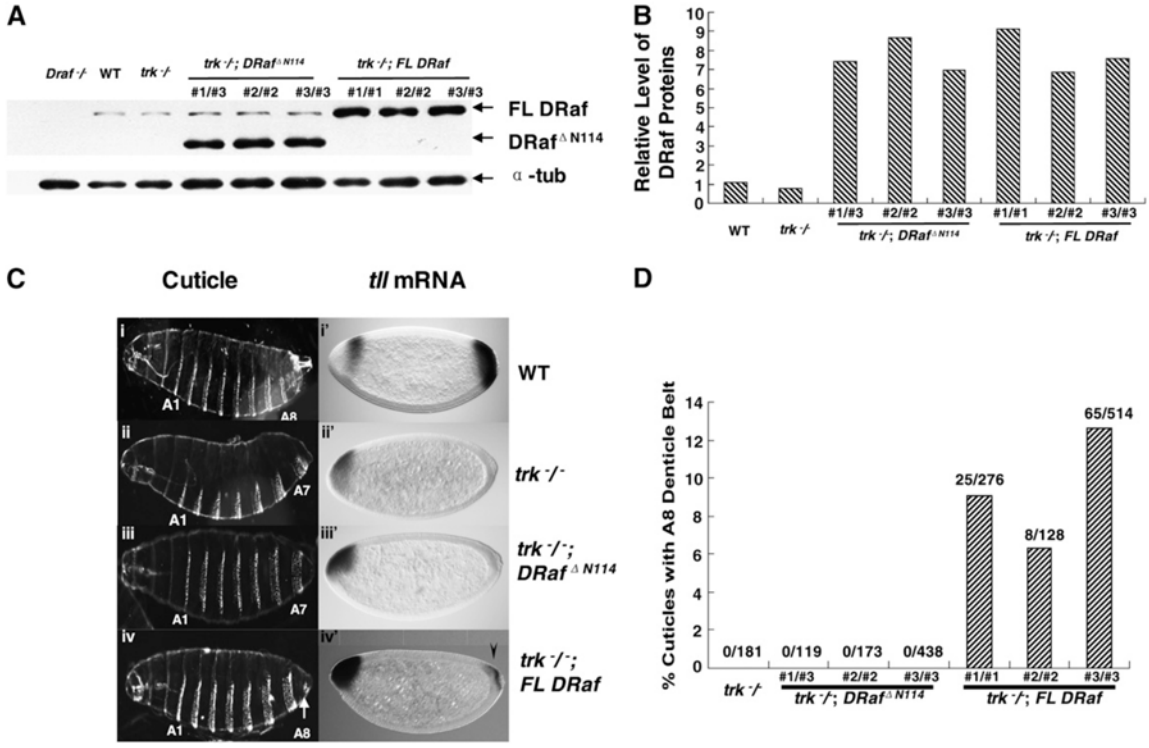

A
Draf -/-
WT
trk -/-
trk -/-; DRaf ΔN114
trk -/-; FL DRaf
#1/#3
#2/#2
#3/#3
#1/#1
#2/#2
#3/#3
FL DRaf
DRaf ΔN114
α -tub
B
Relative Level of DRaf Proteins
WT
trk -/-
trk -/-; DRaf ΔN114
trk -/-; FL DRaf
C
Cuticle
tll mRNA
WT
trk -/-
trk -/-; DRaf ΔN114
trk -/-; FL DRaf
A1
A8
A7
D
% Cuticles with A8 Denticle Belt
0/181
0/119
0/173
0/438
25/276
8/128
65/514

Figure II-5

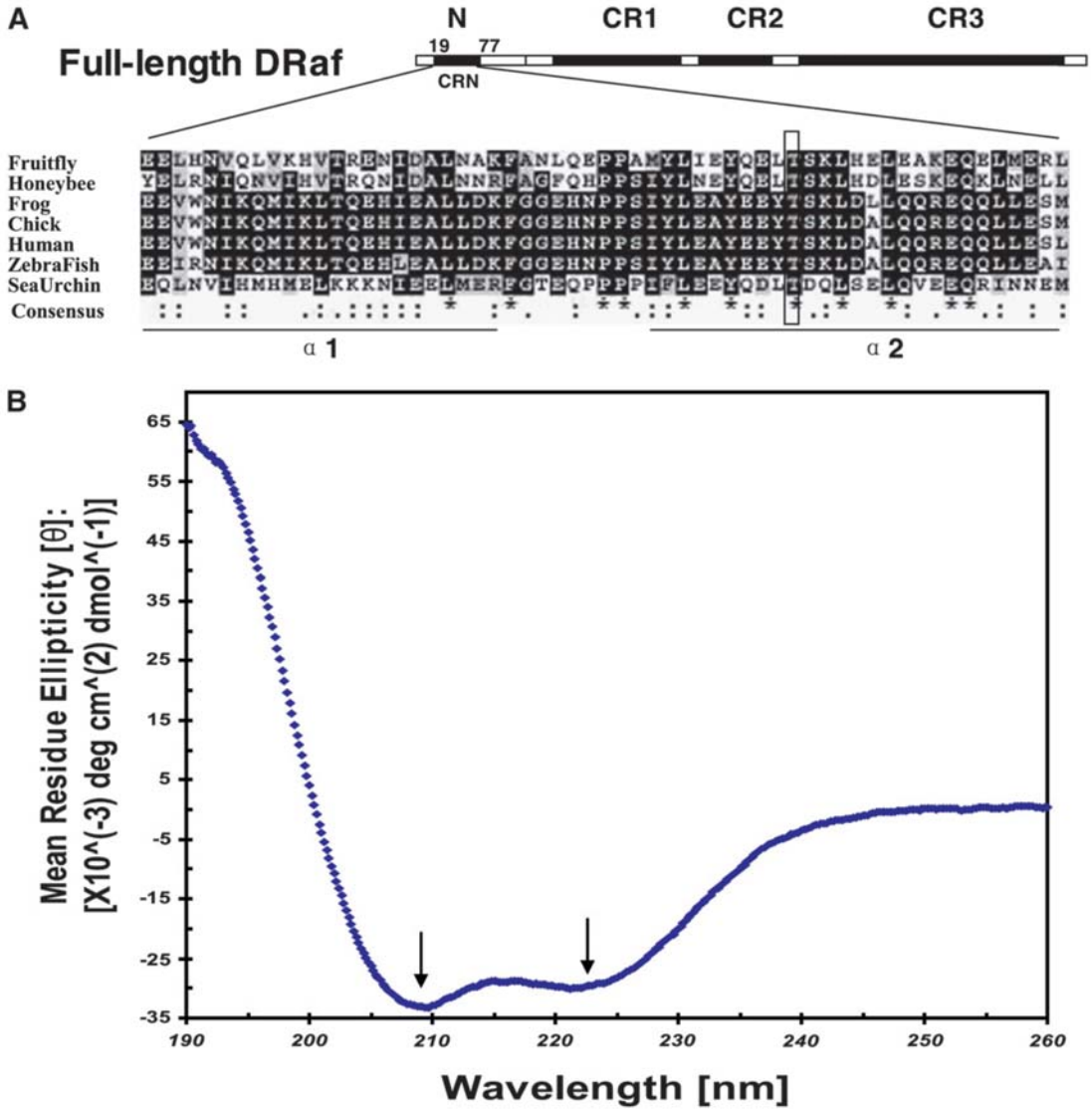
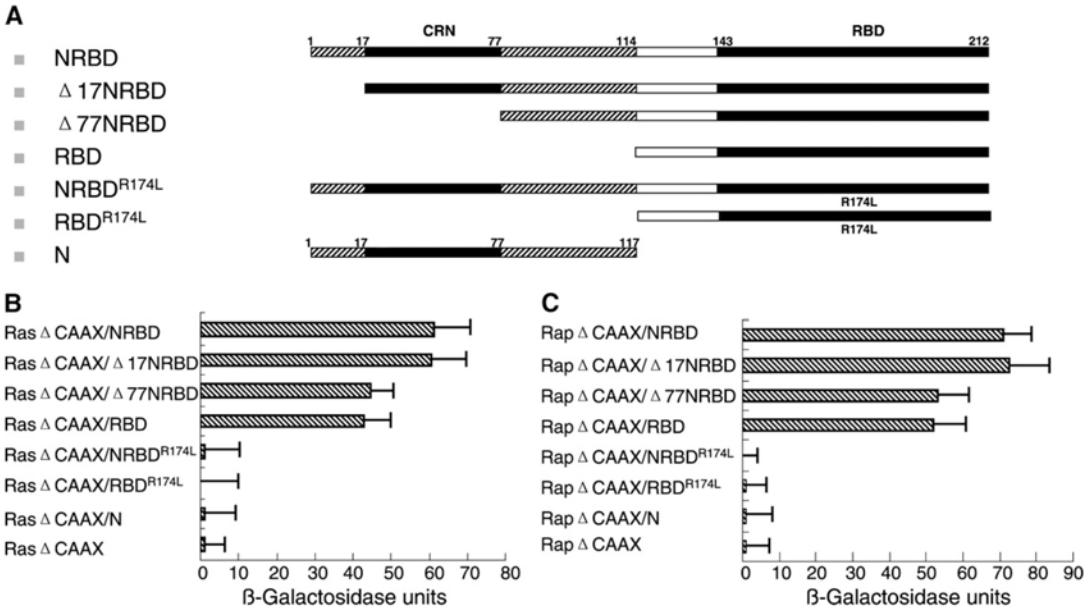

A
Full-length DRaf
N
CR1
CR2
CR3
19
77
CRN
Fruitfly
Honeybee
Frog
Chick
Human
ZebraFish
SeaUrchin
Consensus
α 1
α 2
B
Mean Residue Ellipticity [θ]:
[X10^(-3) deg cm^(2) dmol^(-1)]
65
55
45
35
25
15
5
-5
-15
-25
-35
190
200
210
220
230
240
250
260
Wavelength [nm]

Figure II-6


A
NRBD
Δ17NRBD
Δ77NRBD
RBD
NRBD^R174L
RBD^R174L
N
CRN
RBD
1
17
77
114
143
212
117
R174L
R174L
B
Ras Δ CAAX/NRBD
Ras Δ CAAX/ Δ 17NRBD
Ras Δ CAAX/ Δ 77NRBD
Ras Δ CAAX/RBD
Ras Δ CAAX/NRBD^R174L
Ras Δ CAAX/RBD^R174L
Ras Δ CAAX/N
Ras Δ CAAX
0 10 20 30 40 50 60 70 80
ß-Galactosidase units
C
Rap Δ CAAX/NRBD
Rap Δ CAAX/ Δ 17NRBD
Rap Δ CAAX/ Δ 77NRBD
Rap Δ CAAX/RBD
Rap Δ CAAX/NRBD^R174L
Rap Δ CAAX/RBD^R174L
Rap Δ CAAX/N
Rap Δ CAAX
0 10 20 30 40 50 60 70 80 90
ß-Galactosidase units

Contribution of this work

Unless otherwise noted, the experiments in this paper were performed by Jian Ding. The circular dichroism assay in Figure II-5B was performed by Oren Tchaicheeyan. Jian Ding and Dr. Ambrosio contributed to the writing, reviewing and editing of this work.

CHAPTER 3. Initial Characterization of Drosophila Arf-like 1

Manuscript in preparation

Jian Ding[1], Yukiko Yamada[2], Clark Coffman[2], Linda Ambrosio[1*]
[1] Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa, 50011 and [2] Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa, 50011
*Corresponding author (lima@iastate.edu)

Abstract

The gene encoding Arl1 was originally cloned from Drosophila. Studies on this small GTPase have mainly focused on mammalian and yeast Arl1 proteins and mostly were conducted in cultured cells or *in vitro*. Although it has been demonstrated that Arl1 can recruit a set of golgins to golgi through interaction with the GRIP domain and functions in endosome-to-TGN (*trans*- Golgi Network) trafficking, the roles Arl1 plays in the development of multi-cellular organisms are not well defined. In this study, we describe our strategies to characterize the function(s) of Arl1 in the developmentally tractable system of Drosophila. We found, like its homologues in mammals and yeast, epitope-tagged fly Arl1 was colocalized with Golgi markers both *in vitro* and *in vivo*. We analyzed three different *arl1* mutants in Drosophila, and found that Arl1 is limitedly required during embryogenesis, although its mRNA accumulates ubiquitously in embryos. Most homozygous mutants of Arl1 died during larval-pupa stage, however, we did not detect specific phenotypes associated with lethality. Ubiquitous expression of constitutively active arl1^{Q70L}-mCherry reduced viability of flies, but did not affect development of the eye, wing, leg or bristles. Accumulation of arl1^{Q70L}-mCherry in nurse cells resulted in delay of border cell migration, suggesting that Arl1 may function in cellular morphogenetic movements.

Introduction

The small GTPase ADP-ribosylation factor-like (Arf-like, Arl) proteins have high sequence similarity with Arfs. Among all Arl family members, Arl1 (Arf-like 1) is the closest relative to the Arfs (BURD *et al.* 2004). In spite of some properties similar to ARF proteins, such as the ability to bind and hydrolyze GTP, Arl1 was found completely devoid of ADP ribosylation activity, suggesting Arl1 is not a functional homolog of ARF (TAMKUN *et al*. 1991).

The gene encoding Arf-like 1 (Arl1), was first cloned from *Drosophila* (Arf72A). It appears essential for normal development of flies (TAMKUN *et al*., 1991). Subsequently, Arl1 was identified in mammals and yeast, exhibiting 60~80% amino acid sequence identity with *Drosophila* Arl1 (SCHURMANN *et al*., 1994; LEE *et al*., 1997).

Like other G proteins, Arl1 has two guanine nucleotide binding forms, GTP-Arl1 and GDP-Arl1. The GTP-bound Arl1 is active, while GDP-Arl1 is inactive. In mammals and yeast, active Arl1 is associated with the *trans*- side of Golgi. It seems that association of Arl1 with Golgi is saturable and requires N-terminal glycine myristoylation. A mutant of Arl1 lacking the myristoylation motif (*arl1*^{G2A}, Gly2 Ala2) was absent from the Golgi (LOWE *et al.* 1996; LEE *et al*. 1997; LU *et al.* 2001).

Active Arl1 can recruit a subset of golgins (Golgin-97, Golgin-245, Imp1p, *etc.*) onto Golgi membranes. These golgins have been suggested to function in maintaining the structure of the *trans*- Golgi network (TGN) and play a role(s) in endosome-to-TGN traffic (WU *et al*. 2003; LU *et al*. 2004; YOSHINO *et al*. 2005). The active Arl1 binding site has been mapped into the specific coiled-coil GRIP domains at the C-terminus of these golgins. However, interaction between Arl1 and some of its putative effectors are still controversial.

Burguete *et al*. (2007) found that both Arl1 and another golgi localized small GTPase, Rab6, bound to the C-terminus of golgin GCC185 *in vitro* and may cooperate in recruitment of GCC185 to Golgi membrane. While in the study by Houghton *et al.* (2009), the interaction between Rab6 or Arl1 and GCC185 was not detected, and localization of GCC185 appeared to be independent of Rab6 and Arl1 in culture cells.

In CHO cells, exogenous expression of the GTPase-defective and constitutive active mutant arl1^{Q71L} causes expansion of Golgi. While over-expression of arl1^{T31N}, the dominant negative nucleotide exchange defective mutant of Arl1, leads to disassembly of Golgi apparatus (LU *et al.* 2001). Using a different approach, depletion of Arl1 by RNAi results in redistribution of specific TGN golgins including Goglin245, Golgin97 as well as SNARE proteins Vti1a and syntaxin 6 and 16, suggesting it functions in maintaining Golgi structures and intracellular traffic (NISHIMOTO-MORITA *et al.* 2009).

Overexpression of the GRIP region of golgin97, which is an effector of Arl1, abolished transport of E-cadherin from TGN to plasma membrane in Hela cells (LOCK *et al.* 2005). However, knockdown of Arl1 by RNAi did not change E-cadherin traffic (ZAHN et al. 2008). Although Arl1 was reported to regulate retrogade transport of Shiga toxin to TGN in cultured cells, no specific endogenous cargo was found for Arl1-mediated trafficking machinery so far.

In Drosophila, Eisman *et al*. (2006) identified a GRIP-domain containing protein, centrosomin's beautiful sister (Cbs, CG4840), which might be the homolog of mammalian Golgin-97 based on their sequence similarity. This Cbs protein functions in the centrosome cycle during mitosis potentially in an Arl1-dependent manner in Drosophila embryos and may be a putative effector of Arl1. However, the role Arl1 plays in regulating Cbs, and how

Arl1 functions in development of the multi-cellular organism remains elusive.

In a genome-wide RNAi assay performed by Friedman and Perrimon (2006) on Drosophila S2 cells, Arl1 was found among the candidates, which could be involved in the Ras-Raf-MEK RTK signaling. However, such information from high-throughput screen needs to be confirmed by further analysis and validated *in vivo*.

In this study, genetic analysis of three different *arl1* mutants suggested that Arl1 could function in cell morphogenesis. Although Arl1 was found to interact with DRaf's CR2 region in our yeast two-hybrid assay, it seemed that Arl1 mutations did not alter DRaf signaling. While an essential gene, Arl1 plays limited roles during Drosophila embryogenesis likely due to its functional redundancy with other genes. We also propose potential strategies, which may be helpful for further characterization of Arl1's function.

Materials and Methods

Fly stocks: In this study, Oregon R, $arl1^{4107}$(Bloomington Stock Center, #4107), $arl1^{9.2BC}$, $arl1^{1}$ (obtained from Dr. Jim Kennison) lines were used. The "FLP-DFS" technique was utilized to generate $arl1^{9.2BC}$ and $arl1^{4107}$ germline clones (CHOU and PERRIMON 1996). Drosophila stocks were raised at 25° on standard cornmeal medium.

Molecular Characterization of Mutations: DNAs from $arl1^{9.2BC}$ /+ and $arl1^{4107}$/+ were used as templates for PCR reactions with primers P1-Forward (5' GCGCCA GCACTAGACAAAG 3') and P1-Reverse (5'GAAATAGCACGAATTGAGATTAG 3') that compass the open reading frame (ORF) of the Arl1 gene. PCR products were cloned to the pGEM-T vector. For each allele, 10 colonies were picked up and sequenced.

DNA constructs: The YFP-Golgi construct was kindly provided by Dr. Cungui Mao. T30N and Q70L mutations in Arl1 were generated by PCR-based site-directed mutagenesis and confirmed by sequencing. EGFP-tagged Arl1s were constructed by inserting DNA encoding Arl1 or Δ17*arl1* into the pEGFP-N1 vectors (Clontech). The coding sequence for Myc or mCherry epitopes were fused to the C-termius of *arl1* cDNA inserted into the pMT vector to generate Arl1-Myc or Arl1-mCherry constructs.

Transgenic design: DNA encoding mCherry-tagged wild type Arl1 was cloned into the polylinker site of the *P*-element transformation vector pCaSpeR-HS83. The constitutively active heat-shock 83 gene promoter was used to drive the expression of wild type Arl1-mCherry. The arl1Q70L-mCherry cDNA was cloned into the pUASp vector and used to generate the UASp-arl1Q70L-mCherry construct. The UASp promoter allows spatio-temporal expression when combined with different Drosophila Gal4 drivers. Transgenic lines were generated by *P*-element-mediated transformation.

Transfection in S2 cells: Drosophila S2 cells (obtained from Drosophila Genomics Research Center) were grown in Schneider's medium (Cambrex) supplemented with 10% fetal bovine serum (Invitrogen) at 25 °C. The cultured cells were transfected using CaPi transfection kit according to the standard protocol (Invitrogen). Cells transfected with pMT construncts were treated with 50ug/ml CuSO4, 14 hours after transfection to induce the MT (Metallothionein) promoter.

Immunostaining: Embryos and ovaries were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH7.4) and immunostained with antibodies using standard protocols. Hoechst was used to stain DNA. Mouse-anti-FasIII (1:1000) and Rat-anti-E-Cad (1:200) were obtained from Developmental Studies Hybridoma Bank (Iowa City), and

Rabbit-anti-GFP (1:1000) was from (Torrey Pines Biolabs). Secondary antibodies conjugated with Alexa Fluor were obtained from Molecular Probes.

***In situ* hybrizidation:** *arl1* probes were generated from wild type cDNA clones (NM_079374) using the PCR DIG probe synthesis kit (Roche Applied Science). Whole-mount mRNA *in situ* hybridizations were performed in embryos according to the protocol of Tautz and Pfeifle (1989) with minor modifications.

Yeast two-hybrid: Cbs (NM_137031) cDNA was obtained from the Drosophila Genomics Research Center. DNA sequences corresponding to DRafCR2, Δ17Arl1, $\Delta17Arl1^{T30N}$, $\Delta17Arl1^{Q70L}$, and CbsGRIP proteins were cloned into pGADT7 or pGBKT7 vectors (Clontech). These recombinant constructs in pGADT7 and pGBKT7 plasmids were transformed into yeast Y187 strain according to standard protocol (Clontech). Protein-protein interactions were tested by ß-Galactosidase assays using X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma; solid-support assay) as substrates. All yeast two-hybrid experiments are confirmed by reciprocal bait-prey assays and repeated at least 4 times.

Results

Interaction between Arl1 and DRaf's CR2 region: In our yeast two-hybrid screen using the CR2 region of DRaf as the bait, we obtained a positive colony and subsequently the plasmid Drosophila insert was identified as the small GTPase Arl1 (Table III-1). The interaction between Arl1 and CR2 was confirmed by reciprocal bait-prey assays, suggesting Arl1 may be a binding partner of DRaf and play a role(s) in Raf signaling.

Generation and identification of *arl1* alleles: We initiated our study on Arl1 using a classic genetic strategy to characterize Arl1 phenotypes in *arl1*$^{-}$ mutant animals. A recessive

lethal *arl1* mutation was recovered by Tamkun *et al.* (1991), and it was used to demonstrate that *arl1* is an essential gene. We obtained one loss-of-function allele of *arl1, arl1*^{4107}, from the Bloomington Drosophila stock center (#4107). Generation of an additional loss-of-function allele and phenotypic analysis of these different *arl1* alleles is the strategy we used to characterize Arl1's function in *Drosophila* development. Using *arl1*^{4107}, we conducted a mutagenesis screen to identify non-complementing alleles of *arl1* (See Appendix 1). In our screen (~3,000 chromosomes), we isolated one new allele *arl1*$^{9.2BC}$. When placed over a deficiency for the chromosomal region that covers *arl1* gene [*Df(3L)th102*], both *arl1*^{4107} and *arl1*$^{9.2BC}$ exhibited non-complementation. Both *arl1*^{4107}/*Df(3L)th102* and *arl1*$^{9.2BC}$/*Df(3L)th102* were viable when in a background that contains a duplication for the *arl1* gene (Table III-2). So, it was confirmed that both *arl1*^{4107} and *arl1*$^{9.2BC}$ are loss-of-function alleles of *arl1*. However, neither *arl1*^{4107}/*arl1*^{4107} nor *arl1*$^{9.2BC}$/*arl1*$^{9.2BC}$ can be rescued by the wild type Arl1 transgene obtained from J. Kennison, indicating these two lines contained additional lethal mutations on the mutant *arl1* chromosome. We sequenced the two *arl1* alleles. The *arl1*$^{9.2BC}$ mutation was likely a complete loss-of-function allele, as it was predicted to encode a truncated Arl1 protein of 36 amino acids (Figure III-1). The equivalent change for mammalian Arl1 would eliminate the effector and all known protein interaction domains (WU *et al*. 2003). The *arl1*^{4107} mutation was predicted to encode a truncated Arl1 protein of 165 amino acids, lacking the C-terminal 15 amino acids (Figure III-1), this region is known to form an α-helical (α5) structure in mammalian Arl1 (WU *et al*. 2003).

Classical genetic analysis of Arl1 using *arl1*$^{-}$ mutants: Both *arl1*^{4107} and *arl1*$^{9.2BC}$ are recessive lethal mutations. We found most homozygous *arl1*^{4107} or *arl1*$^{9.2BC}$ mutants proceeded through embryogenesis and died during larval and pupal stages (Table III-3).

Cuticles of unhatched of *arl1*$^{-}$/*arl1*$^{-}$ embryos appeared to be relatively normal with 1-2% showing head involution defects (HID) (Table III-4). All *arl1*$^{-}$/*arl1*$^{-}$ larvae appeared normal in their exoskeletal pattern. Using a different allele, *arf72A*^{1} (*arl1*^{1}), Eisman *et al.* (2006) reported that loss of Arl1 function led to mitotic defects and embryonic lethality. Subsequently, we requested and obtained the *arl1*^{1} line from the Kennison's group, who originally isolated the *arl1*^{1} allele. Like *arl1*^{4107} or *arl1*$^{9.2BC}$ alleles, *arl1*^{1} is recessive lethal. However, most eggs homozygous for the *arl1*^{1} mutation hatched as larva. Embryonic cuticles of homozygous *arl1*^{1} mutants appeared relatively normal (Table III-3, Table III-4). It seems that the *arl1*^{1} chromosome also contains mutations in other genes, for *arl1*^{1}/*Df(3L)th102*, but not *arl1*^{1}/*arl1*^{1} mutants, can be rescued by the wild type Arl1 transgene. Therefore, our genetics analysis using three independent alleles suggests that zygotic Arl1 may be not essential for the development of embryos.

Next we generated females with *arl1*$^{9.2BC}$/*arl1*$^{9.2BC}$ or *arl1*^{4107}/*arl1*^{4107} germ cells to understand Arl1's maternal role. Using the FLP-FRT system (CHOU and PERRIMON 1996), *arl1*$^{-}$/*arl1*$^{-}$ germ line clones were produced and used to study early fly development in the absence of maternal Arl1 activity. When crossed with Oregon R (OR) wild type males, the *arl1*$^{-}$ containing germ line clone virgin females laid eggs, which are zygotically heterozygous for Arl1, but lacked maternal Arl1 activity. Some eggs derived from *arl1*$^{-}$/*arl1*$^{-}$ germ cells did not hatch. Most embryonic cuticles of the unhatched eggs are phenotypically normal (Table III-5, Table III-6), and all have the posterior Filzkörper structure, and normal central abdominal development suggesting the Torso RTK-Ras-Raf signaling pathway is not affected by the *arl1* mutation (Loss of Torso RTK signaling results in loss of the Filzkörper, while gain of pathway activity causes reduced segmentation in the central abdominal region).

About 70% of those unhatched eggs do not have cuticles and are likely unfertilized, since most embryos (0-15 hours after egg deposition), when stained with a nucleus marker and the FasIII antibody, appeared to be phenotypically normal with only a few showing head involution defects (Table III-6, 7; Figure III-2). The genetic cross with *arl1*$^{-}$/+ males did not significantly alter the hatch rates of the eggs from the females with *arl1*$^{-}$/*arl1*$^{-}$ germ lines (Table III-5), implying removal of paternal Arl1 in half of the eggs did not affect their embryogenesis. Therefore, our study suggests both maternal and zygotic Arl1 proteins play limited roles during embryogenesis.

In our genetic assays, head involution defects were observed for some embryos, although at low penetrance. Thereby, loss of Arl1 function may lead to abnormal cellular morphogenetic movements. The HID defect may not be relevant to Ras-Raf signaling, since we did not detect a genetic interaction, when the *arl1*$^{-}$/*arl1*$^{-}$ germline-bearing females were crossed with *ras1*^{e2b}/+ males (Table III-6).

The head involution defects observed could be due to the dysregulation of adhesion proteins (VANHOOK and LETSOU 2007). Adhesion molecules are thought to function in cellular morphogenesis through their dynamic presence on the cell surface, which can be regulated by endocytosis and exocytosis (EDELMAN 1985; GUMBINER 1996; SETO *et al.* 2002). Considering Arl1's potential roles in trafficking, we developed the hypothesis that Arl1 might participate in intracellular transport of adhesion proteins. If so, then in eggs from *arl1*$^{-}$/*arl1*$^{-}$ germ cells when fertilized by males with a mutation in a gene that acts in adhesion, reduced viability is anticipated for transheterozygous individuals. We tested for genetic interaction of *arl1* alleles with mutations in numerous genes encoding membrane-localized adhesion molecules. However, no additive or synergistic effects were seen (Table III-8, 9).

Many coiled-coil golgin tethers, containing GRIP domains, have been identified as Arl1 effectors. The Golgi localization of these golgins, likely mediated by the interactions between the GRIP domains and Arl1, appeared essential for their functions (LU *et al.* 2004). However, recently these golgins have been shown to associate with numerous additional small GTPase regulators. For instances, dGolgin97, dGolgin245 and dGCC185 contain multiple binding sites for the Rab family of G proteins (SINKA *et al.* 2008). Therefore, Arl1 may act, together with these Rab proteins, to mediate the function of downstream golgins. This implies a degree of redundancy between the Arl1 and Rabs, which could explain only mild phenotypes observed in *arl1* mutants upon removal of normal Arl1 activity. However, no genetic interaction, with the limited number of candidates (Rab6, Rab11 *etc.*) that we tested, was detected (Table III-10, 11). Therefore, there might be other proteins that function redundantly with Arl1. Together, our analysis implies that it is difficult to dissect Arl1's roles using classical genetic methods to study these *arl1* alleles.

An over-expression strategy to characterize Arl1's function in Drosophila: One strategy for characterizing a gene's functions is to study the effects of overexpressing its mutant products. In mammalian cultured cells, over-expression of constitutive active arl1^{Q71L} abolishes traffic of VSVG and Shiga toxin (LU *et al*. 2001). We generated the mCherry-tagged arl1^{Q70L} with an equivalent point mutation. The arl1^{Q70L}-mCherry cDNA was cloned into pUASp vector and used to generate UASp-arl1^{Q70L}-mCherry transgenic flies. The UASp promoter allows spatio-temporal expression when combined with different Drosophila Gal4 drivers in somatic and germ-line cells (RORTH 1998). We also generated the wild type Arl1-mCherry lines as the control. The constitutively active heat-shock 83 (HS83) promoter was selected to drive the ubiquitous expression of mCherry-tagged Arl1 protein (pCaspeR-HS83-

Arl1-mCherry). Overexpression of Arl1-mCherry did not affect the viability of the flies, and the Arl-mCherry transgene rescued the lethality of *arl1^{4107}/Df(3L)th102* and *arl1$^{9.2BC}$/Df(3L)th102* flies, suggesting mCherry-tagged Arl1 is capable of replacing the endogenous gene.

We found ubiquitous expression of arl1^{Q70L}-mCherry driven by actin-Gal4 or tubulin-Gal4 decreased the viability of flies. However, development of eyes, wings, legs and thoracic bristles of surviving adults appeared phenotypically normal. Considering the potential roles of Arl1 in traffic, we also tried to over-express arl1^{Q70L}-mCherry using the salivary gland-Gal4 (SG-Gal4) driver to see if it affects secretion of glue proteins and/or the morphology of salivary gland cells. However, we did not detect any defects for *SG-Gal4/+; arl1^{Q70L}-mCherry* larva and their salivary glands appeared normal when stained with E-cadherin and a nuclear marker. All the these larvae stick to the vial wall and form pupa as well as wild type, suggesting secretion of glue proteins was relatively normal.

Expression of arl1^{Q70L}-mCherry in ovaries: During oogenesis, border cells migrate between nurse cells to the anterior end of oocyte. Cell surface-localized adhesion molecules mediate the border-nurse cell contacts required for this process. Their localization on plasma membranes can be regulated dynamically by exocytosis and endocytosis, so that rounds of binding and release allow the border cells to move between the nurse cells (MONTELL 2003; BRYANT and STOW 2004). Dysregulation of the dynamic presence of adhesion proteins on the cell surface could result in border cell migration defects, which subsequently leads to a failure of micropyle structure formation and female sterility. We found over-expression of arl1^{Q70L}-mCherry in germ cells driven by nanos-Gal4 reduced female fertility and ~8% of stage 10 egg chambers of these females exhibited a delay in border cell migration (Figure III-

3, Table III-12). We did not detect such migration defects upon expression of arl1^{Q70L}-mCherry driven by slbo-Gal4 in border cells. Considering Arl1's potential functions in intracellular transport, we propose that the border cell migration defects upon over-expression of arl1^{Q70L}-mCherry might be due to dysregulation of adhesion molecules in nurse cells. However, none of the mutations in adhesion molecules we tested showed additive or synergistic effects when arl1^{Q70L}-mCherry was over-expressed in germ cells (Table III-13).

Accumulation of Arl1 mRNA in Drosophila embryos: The spatio-temporal expression pattern of a gene sometimes exhibits correlation with its function(s). A study of the Arl1 mRNA accumulation pattern in embryos may provide some clues about its biological roles. We performed whole mount *in situ* hybridization to explore when and in which cells the Arl1 gene is transcribed during embryogenesis. We found Arl1 mRNA was ubiquitously distributed in embryos with a higher level in certain tissues. Arl1 mRNAs were enriched underneath the cortical layer of stage 5 embryos. At later stages (stage 6-10), abundant Arl1 mRNAs were detected in the ventral, cephalic, anterior and posterior transversal furrows. After completion of gastulation, Arl mRNAs were enriched in brain lobes and central nerve system (Figure III-4).

Epitope-tagged Arl1 is colocalized with Golgi marker: In yeast and mammalian cultured cells, Arl1 was found localized in Golgi membrane (LOWE *et al.* 1996; LEE *et al*. 1997; LU *et al.* 2001). To study the subcellular localization of Arl1, Drosophila S2 cells expressing different epitope-tagged (mCherry, Myc and EGFP) Arl1 proteins were co-transfected with YFP-tagged Golgi marker (YFP-Golgi, containing the N-terminal 1-81 region of human ß 1,4-galactosyltransferase, which is a *trans*- Golgi protein) or stained with the Golgi marker TRITC-Ceramide (LAJEUNESSE *et al*. 2004). All these epitope-tagged Arl1

proteins exhibit a punctuate pattern and show nice colocalization with Golgi markers (Figure III-5). Arl1's localization *in vivo* was also tested using the Arl1-mCherry transgenic lines. We dissected the ovaries from females *YFP-Golgi/Arl1-mCherry* and found Arl1-mCherry is colocalized with the Golgi marker in oocytes. Consistently, Arl1-mCherry also showed a pattern of colocalization with YFP-Golgi in brain cells from the 3rd instar larva of the transgenic lines (Figure III-6).

In mammals, the N-term 1-17 region is very likely to form an amphipathic helix and contains a glycine site (G2), which can be myristoylated. The glycine myristoylation and probably the amphipathic helix structure, are important for targeting Arl1 to Golgi membranes (LU *et al*., 2001; WU *et al*., 2003). Deletion of the N1-17 region or mutation at G2 results in loss of golgi localization. In our study, when the N-terminal 1-17 region was removed (Δ17arl1-EGFP), Arl1 lost its fragmented pattern and instead was dispersed in the cytoplasm (Figure III-5B). These findings in Drosophila are consistent with those in yeast and mammals.

Interaction between Arl1 and GRIP: Previously, Eisman *et al.* (2006) identified a GRIP domain-containing protein, centrosomin's beautiful sister (Cbs, CG4840). Cbs apprears to be the ortholog of golgin97, and is a putative effector of Arl1. We found that Arl1 can associate with the GRIP region of Cbs in a GTP dependent manner in a yeast two-hybrid assay. The coiled-coil region (CC) preceding GRIP contains binding sites for small GTPase Rab proteins, however, we did not detect interaction between CC and Arl1 (Figure III-7). These are consistent with previous studies by others (SINKA *et al.* 2008).

Discussion

Is Ar1l involved in DRaf signaling: In our yeast two-hybrid screen using the CR2 region of DRaf as the bait, we identified the small GTPase Arl1 as a potential binding partner of DRaf. In yeast and mammalian cultured cells, the Golgi-localized Arl1 can interact with the GRIP domain of coiled-coil golgin proteins, and is thought to function in intracellular transport (LOWE *et al.* 1996; LEE *et al*. 1997; LU *et al.* 2001; LU *et al.* 2004). Although mammalian Raf has been found associated with Golgi, this was attributed to Ras binding (CHIU *et al.* 2002). The interaction between Arl1 and Raf has not been described, and it is not clear if Arl1 is relevant to Raf signaling.

Friedman and Perrimon (2006) performed a genome-wide RNAi assay in Drosophila S2 cells to identify potential signal components of RTK pathways. In their primary screen, knock down of Arl1 seemed to increase the phosphorylation level of ERK, suggesting that Arl1 may have inhibitory effects on RTK signaling. However, such information from high-throughput screen needs to be confirmed by further analysis. Particularly, genetic characterization of Arl1 *in vivo* is necessary for validation of its potential roles.

Previously, a golgi protein, RKTG, has been identified as a negative regulator of CRaf and BRaf in mammalian cells. Over-expression of RKTG can prevent Raf activation by recruiting cytoplasmic Raf to golgi and hidering its translocation to plasma membrane (FENG *et al.* 2007). If Arl1 functions in inhibiting the ERK signal, as suggested by Friedman and Perrimon's study (2006), it is possible that it acts like RKTG to capture Raf on golgi membrane through its interaction with CR2, thus playing a negative role in Raf signaling.

We attempted to determine the function of this small GTPase Arl1 in Drosophila using classic genetic methods. However, we did not detect Raf-related phenotypical effects upon

mutation of Arl1, thus, it is still unclear whether Arl1 is relevant to ERK signal transduction *in vivo*.

Arl1 may function in cellular morphogenetic movements: Adhesion molecules, like cadherin proteins, play very important roles in epithelial morphogenesis, cell-cell communications, as well as cell migration. In order to fulfill these roles, these adhesion proteins must be delivered efficiently to the cell surface, where they are incorporated into protein complexes with other partners (EDELMAN 1985; GUMBINER 1996). During cell migration, their localization to the plasma membrane can be regulated dynamically by exocytosis and endocytosis (SETO *et al.* 2002; BRYANT and STOW 2004). Dysregulation of their dynamic presence on the cell surface could result in morphogenesis defects. In our study, over-expression of arl1^{Q70L}-mCherry in germ cells resulted in a delay of border cell migration in some egg chambers. We also observed, though at low penetrance, head involution defects in embryos with Arl1 mutations. These suggest Arl1 may function in cellular morphogenetic movements. Considering Arl1's golgi localization and its potential role in intracellular transport, it is possible that Arl1 may be involved in trafficking of adhesion molecules. For instance, loss of normal Arl1 function could inhibit the endocytosis of adhesion molecules in nurse cells. Therefore the constitutive presence of the "sticky" adhesion proteins on the cell surface would hider the movement of border cells and result in delay of their migration.

Dynamic rearrangement of the cytoskeleton, including actin filaments, also contributes to cellular morphogenesis. Distorting the actin cytoskeleton in Drosophila embryos results in head involution/dorsal closure defects (ELISABETH and MULLER 1998). Previously, it has been reported that some small GTPases (Rac, Rho, *etc.*) can regulate the structure of the actin

cytoskeleton (SHARP *et al*. 2005). It is also possible that Arl1 could be involved in cellular morphogenetic movement, like these small G proteins, by regulating dynamic rearrangement of cytoskeleton.

Challenges in genetic analysis of Arl1's developmental roles: The Arl1 small GTPase is thought to regulate coiled-coil golgin proteins through interaction with their GRIP domains (LU *et al.* 2004). The golgins may act like tethers and provide the contact sites for other golgi cisternae, arriving cargo, and intracellular vesicles including endosomes, thereby function in golgi morphology and traffic. However, these golgins contain binding sites for other regulators, in addition to Arl1. Particularly, the Golgi-localized Rab small GTPases (Rab2, Rab6, Rab19, Rab30, Rab39, *etc.*) can interact with numerous coiled-coil golgin tethers and may function redundantly with Arl1 (SINKA *et al.* 2008). This may explain why loss of Arl1 activity only resulted in mild phenotypes in Drosophila embryos.

In mammals, there are ~70 members of the Rab small GTPase family. These Rab proteins have been found localized in different intracellular organelles (ER, Golgi, early endosome, late endosome, recycling endosome, *etc.*) and function in membrane trafficking (STENMARK and OLKKONEN 2001). In Drosophila, 33 Rab members with high sequence similarity have been identified (ZHANG *et al.* 2007). The golgin proteins contain multiple binding sites in their coiled-coil regions for numerous Rab proteins, which may include, besides those localized in Golgi, some associating with other intracellular vesicles. It is possible, as proposed by SINKA *et al.* (2008), that golgins may be anchored to the Golgi through interactions between their C-termini and Arl1 or Rab6 *etc.*, while the remaining portions of these molecules may project into the cytoplasm like tentacles. Numerous coiled-coil golgin proteins (Golgin97, Golgin245, *etc.*) share this structural feature with mutiple

binding sites for Rab members located along the tentacles (SINKA *et al.* 2008), so that they could act collectively by capturing arriving vesicles bearing Rabs (Rab4, Rab5, Rab9, or Rab11-bearing endosomes, *etc.*) to Golgi membranes. If so, the GRIP-containing golgin proteins, which are potential effectors of Arl1, could also function redundantly with each other. This could explain why, in several recent studies, functional depletion of particular golgins did not lead to severe phenotypic defects (SINKA *et al.* 2008). Together, the potential functional redundancy for Arl1 as well as its effectors may confound our genetic analysis on Arl1.

Potential strategies for further characterization of Arl1's role: Drosophila has been thought to be one of the best-understood multi-cellular model organisms. However, among more than 15,000 annotated genes in flies, most (~85%), when mutated, did not show detectable phenotypes. Lack of phenotypic information regarding these genes does not mean they don't have significant functions, instead, it is likely that most of them play important roles but act redundantly with other genes (www.flyrnai.org/TriP-HOME.html). To resolve such an issue of functional redundancy, one could disrupt the activity of all/most of the redundant genes that play a role in a particular cellular process. NIH has just begun to support TRiP (Transgenic RNAi Project), a project to develop the UAS-RNAi system in a genome-wide scale. By combining RNAi sequences of several different genes (Arl1, Rab2, Rab6, *etc.*), we could simultaneously deplete their functions in flies. Particularly, based on their mRNA pattern in embryos, we could knock down these genes in specific tissues or at specific developmental stages using the Gal4-UAS system, and test their functional roles with a spatial and temporal resolution.

In our study, over-expression of arl1^{Q70L}-mCherry in germ cells resulted in delay of border cell migration in some egg chambers. We also observed, though at low penetrance, head involution defects in embryos that lack maternal Arl1 activity. These findings suggest Arl1 may function in cellular morphogenetic movements. Considering Arl1's golgi localization and its potential role in intracellular transport, we propose that Arl1 may be involved in trafficking of adhesion molecules, although we did not detect any additive or synergistic genetic interaction between Arl1 and numerous genes encoding adhesion proteins. Identification of the components/cargo of the Arl1 complex/traffic machinery using proteomic approaches could be a helpful approach to characterize its developmental roles. Particularly, one could co-precipitate or pull-down the proteins of Arl1 complexes from cellular lysates, and using mass spectrum analysis, identify components of the complex(es). This method may also validate Raf-Arl1 interaction *in vivo*. A combination of such proteomic approaches may allow the *in vivo* genetic analysis of Arl1 more promising.

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Tables

TABLE III-1. Interaction between Arl1 and the CR2 region of DRaf.

Interact w/	N	CR1	CR2	CR3	CR1CR2	NCR1CR2	FL DRaf
Arl1	No	No	Yes	No	Yes	Yes	No

TABLE III-2: Both *arl1*^{4107} and *arl1*$^{9.2B}$ alleles are loss of function alleles of *arl1*.

Allele tested	*arl1*^{4107}	*arl1*$^{9.2Bc}$	*Df(3L)th102*	*Df(3L)st-f13*	*Df(3L)brm11*	*Df(3L)th102; Dp(3;Y)L131-D3*
arl1^{4107}	L	L	L	V	L	V = males
arl1$^{9.2BC}$	L	L	L	V	L	V = males

L = no complementation, lethal; V = viable
Bloomington #3641: *Df(3L)th102, h[1] kni[ri-1] e[s]/TM6C, cu[1] Sb[1] ca[1]*
Breakpoints: 71F3-5;72D12
Bloomington #2993: *Df(3L)st-f13, Ki[1] rn[roe-1] p[p]/TM6B, Tb[1]*
Breakpoints: 72C1-D1;73A3-4
Bloomington #3640: *Df(3L)brm11/TM6C, cu[1] Sb[1] ca[1]*
Breakpoints: 71F1-4;72D1-10
Bloomington #5462: *Df(3L)th102, h[1] kni[ri-1] e[s]/TM6C, cu[1]Sb[1]ca[1]; Dp(3;Y)L131-D3, B[S]*
Breakpoints: 72A2;72D10, 72A;75D4-5;Y

TABLE III-3. Most homozygous *arl1* mutants proceeded through embryogenesis.

Maternal	Paternal	#Hatched (%)	#Unhatched* (%)	Total
arl1$^{9.2BC}$/+	*arl1*$^{9.2BC}$/+	655 (75.1)	217 (24.9)	872
arl1$^{9.2BC}$/+	+/+	627 (87.1)	93 (12.9)	720
arl1^{4107}/+	*arl1*^{4107}/+	482 (79.8)	123 (20.2)	605
arl1^{4107}/+	+/+	355 (87.9)	49 (12.1)	404
arl1^{1}/+	*arl1*^{1}/+	201 (83.1)	41 (16.9)	242
arl1^{1}/+	+/+	177 (89.4)	22 (10.6)	198
arl1^{1}/+	*arl1*$^{9.2BC}$/+	213 (84.9)	38 (15.1)	251
+/+	+/+	347 (86.3)	55 (13.7)	402

* Unhatched eggs comprise three classes: unfertilized eggs, embryos that develop poorly and do not make cuticles and embryos that produce cuticles but do not hatch.

TABLE III-4. Embryonic cuticles of *arl1⁻/arl1⁻* mutants are relatively normal.

Maternal	Paternal	Non-GFP embryos		Total
		#Normal (%)	#HID *(%)	
arl1$^{9.2BC}$/GFP	*arl1$^{9.2BC}$/GFP*	71 (98.6)	1 (1.4)	72
arl1^{4107}/GFP	*arl1^{4107}/GFP*	88 (98.8)	1 (1.2)	89
arl1^{1}/GFP	*arl1^{1}/GFP*	60 (100)	0 (0)	60
arl1$^{9.2BC}$/arl1$^{9.2BC}$	*arl1$^{9.2BC}$/GFP*	25 (92.6)	2 (7.4)	27

* HID: Head involution defects

TABLE III-5. Hatch rates of eggs from germline clone bearing females.

Maternal	Paternal	#Hatched (%)	#Unhatched * (%)	Total
arl1$^{9.2BC}$/arl1$^{9.2BC}$	*arl1$^{9.2BC}$*/+	203 (60.4)	133(39.6)	336
arl1$^{9.2BC}$/arl1$^{9.2BC}$	+/+	456 (63.3)	264(36.7)	720
arl1^{4107}/arl1^{4107}	*arl1^{4107}*/+	287 (41.8)	400 (58.2)	687
arl1^{4107}/arl1^{4107}	+/+	372 (33.8)	728 (66.2)	1100
FRT2A/FRT2A	+/+	146 (70.9)	60 (29.1)	206

* Unhatched eggs comprise three classes: unfertilized eggs, embryos that develop poorly and do not make cuticles and embryos that produce cuticles but do not hatch.

TABLE III-6. Some embryonic cuticles of unhatched eggs from germline clone bearing females have head involution defects or body hole.

Maternal	Paternal	#Normal (%)	#HID or BD* (%)	#Total
arl1$^{9.2BC}$/arl1$^{9.2BC}$	+/+	32 (94.1)	2 (5.9)	34
arl1$^{9.2BC}$/arl1$^{9.2BC}$	*arl1$^{9.2BC}$*/+	29 (93.5)	2 (6.5)	31
arl1^{4107}/arl1^{4107}	+/+	31 (96.9)	1 (3.1)	32
arl1^{4107}/arl1^{4107}	*arl1^{4107}*/+	21 (95.5)	1 (4.5)	22
arl1$^{9.2BC}$/arl1$^{9.2BC}$	*Ras^{e2B}*/+	25 (96.2)	1 (3.8)	26
FRT2A/FRT2A	+/+	27 (100)	0 (0)	27

*HID or BD: head involution defects or body hole

TABLE III-7. Some embryonic from germline clone bearing females have head involution defcts (FasIII staining).

Maternal	#Normal(%)	#HID*(%)	#Total
arl1$^{9.2BC}$/arl1$^{9.2BC}$	71 (92.2)	6 (7.8)	77
arl1^{4107}/arl1^{4107}	54 (94.7)	3 (5.3)	57
FRT2A/FRT2A	34 (100)	0 (0)	34

*HID: head involution defects

TABLE III-8. Genetic interaction tests between *arl1* mutations and alleles of adhesion genes on the second chromosome (*Cyo: Balancer chromosome**).**

Genetic cross	#CyO *	#Non-CyO	#Total
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X + /CyO;+/+	371	444	815
+/+; $arl1^{4107}/arl1^{4107}$ X +/CyO;+/+	146	179	325
+/+; FRT2A/FRT2A X +/CyO;+/+	320	364	684
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X shg^{k03401}/CyO;+/+	169	125	294
+/+; $arl1^{4107}/arl1^{4107}$ X shg^{k03401}/CyO;+/+	113	103	216
+/+; FRT2A/FRT2A X shg^{k03401}/CyO;+/+	95	89	184
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X shg^{R69} /CyO;+/+	222	204	426
+/+; $arl1^{4107}/arl1^{4107}$ X shg^{R69} /CyO;+/+	58	54	112
+/+; FRT2A/FRT2A X shg^{R69} /CyO;+/+	152	134	286
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X $dystroglycan^{-}$ /CyO;+/+	82	86	168
+/+; $arl1^{4107}/arl1^{4107}$ X $dystroglycan^{-}$ /CyO;+/+	28	37	65
+/+; FRT2A/FRT2A X $dystroglycan^{-}$ /CyO;+/+	150	129	279
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X dg^{B148} /CyO;+/+	61	62	123
+/+; $arl1^{4107}/arl1^{4107}$ X dg^{B148} /CyO;+/+	37	43	80
+/+; FRT2A/FRT2A X dg^{B148} /CyO;+/+	26	32	58
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X ft^{g-rv} / CyO;+/+	63	102	185
+/+; $arl1^{4107}/arl1^{4107}$ X ft^{g-rv} /CyO;+/+	38	46	84
+/+; FRT2A/FRT2A X ft^{g-rv} /CyO;+/+	40	61	101
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X $stan^{192}$ /CyO;+/+	63	73	136
+/+; $arl1^{4107}/arl1^{4107}$ X $stan^{192}$ /CyO;+/+	77	79	156
+/+; FRT2A/FRT2A X $stan^{192}$ /CyO;+/+	56	64	120
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X $l(2)gl^{4}$ /CyO;+/+	29	38	67
+/+; $arl1^{4107}/arl1^{4107}$ X $l(2)gl^{4}$ /CyO;+/+	23	31	54
+/+; FRT2A/FRT2A X $l(2)gl^{4}$ /CyO;+/+	30	40	70
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X ds^{38k} /CyO;+/+	26	34	60
+/+; $arl1^{4107}/arl1^{4107}$ X ds^{38k} /CyO;+/+	24	28	52
+/+; FRT2A/FRT2A X ds^{38k} /CyO;+/+	26	28	54
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X $cadN2^{-}$ /CyO;+/+	43	31	74
+/+; $arl1^{4107}/arl1^{4107}$ X $cadN2^{-}$ /CyO;+/+	24	33	57
+/+; FRT2A/FRT2A X $cadN2^{-}$ /CyO;+/+	22	22	44
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X $cadN^{-}$ /CyO;+/+	17	19	36
+/+; $arl1^{4107}/arl1^{4107}$ X $cadN^{-}$ /CyO;+/+	24	32	56
+/+; FRT2A/FRT2A X $cadN^{-}$ /CyO;+/+	19	24	43
+/+; $arl1^{9.2BC}/arl1^{9.2BC}$ X $cadN^{M12}$ /CyO;+/+	44	31	75
+/+; $arl1^{4107}/arl1^{4107}$ X $cadN^{M12}$ /CyO;+/+	34	28	62
+/+; FRT2A/FRT2A X $cadN^{M12}$ /CyO;+/+	30	25	55

TABLE III-9. Genetic interaction tests between *arl1* alleles and mutations in adhesion genes on the 3rd chromosome (*Tm3: Balancer chromosome)**.**

Genetic cross	#Tm3*	#Non-Tm3	#Total
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+; $cad74A^{-}$/Tm3	90	87	177
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+; $cad74A^{-}$ /Tm3	106	97	203
+/+; FRT2A/FRT2A X +/+; $cad74A^{-}$/Tm3	103	73	176
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+;$fat2^{-}$/Tm3	62	53	115
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+;$fat2^{-}$/Tm3	19	23	42
+/+; FRT2A/FRT2A X +/+;$fat2^{-}$/Tm3	68	53	121
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+; $scrib^{j7B3}$ /Tm3	63	53	116
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+; $scrib^{j7B3}$/Tm3	23	21	44
+/+; FRT2A/FRT2A X +/+; $scrib^{j7B3}$/Tm3	124	131	255

TABLE III-10. Genetic interaction test between arl1 alleles and mutations in candidate genes (2nd chromosome), which may act redundantly with Arl1 (*Cyo: Balancer chromosome) .

Genetic Cross	#CyO*	#Non-CyO	#Total
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X + /CyO;+/+	371	444	815
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/CyO;+/+	146	179	325
+/+; FRT2A/FRT2A X +/CyO;+/+	320	364	684
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X $rab6^{D23D}$/CyO;+/+	111	128	239
+/+; $arl1^{4107}$/$arl1^{4107}$ X $rab6^{D23D}$/CyO;+/+	57	61	118
+/+; FRT2A/FRT2A X $rab6^{D23D}$/CyO;+/+	34	35	69
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X $rab6^{08323}$/CyO;+/+	44	53	97
+/+; $arl1^{4107}$/$arl1^{4107}$ X $rab6^{08323}$/CyO;+/+	32	39	71
+/+; FRT2A/FRT2A X $rab6^{08323}$/CyO;+/+	53	60	113

TABLE III-11. Genetic interaction test between arl1 alleles and mutations in candidate genes (3rd chromosome), which may act redundantly with Arl1 or or be involved in post-translational modification of redundant gene products (*Tm3: Balancer chromosome)**.**

Genetic cross	#Tm3*	#Non-Tm3	#Total
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+; $arf79F^{-}$/Tm3	136	203	339
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+; $arf79F^{-}$/Tm3	51	74	125
+/+; FRT2A/FRT2A X +/+; $arf79F^{-}$/Tm3	66	95	161
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+;$rab11^{93bj}$/Tm3	154	225	379
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+; $rab11^{93bj}$/Tm3	60	80	140
+/+; FRT2A/FRT2A X +/+; $rab11^{93bj}$/Tm3	193	214	407
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+; $rab11^{j2d}$/Tm3	119	179	298
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+; $rab11^{j2d}$/Tm3	65	93	158
+/+; FRT2A/FRT2A X +/+; $rab11^{j2d}$/Tm3	132	198	330
+/+; $arl1^{9.2BC}$/$arl1^{9.2BC}$ X +/+; $rabGGT^{-}$/Tm3	62	73	135
+/+; $arl1^{4107}$/$arl1^{4107}$ X +/+; $rabGGT^{-}$/Tm3	27	23	50
+/+; FRT2A/FRT2A X +/+; $rabGGT^{-}$/Tm3	83	85	168

TABLE III-12. Some egg chambers have border cell migration defects upon over-expression of $arl1^{Q70L}$-mCherry germ cells.

Maternal	# w/ Border Cell migration defects (%)	#Normal (%)	#Total
Arl1-mCherry	0 (0)	32 (100)	32
$arl1^{Q70L}$-mCherry	4 (7.8)	47 (92.2)	51

TABLE III-13. Genetic interaction tests of mutations in adhesion genes or candidate genes, which may act redundantly with Arl1 (border cell migration assay).

Alleles	Normal gene products	Genetics interaction with arl1^{Q70L}-mCherry ?
Rab6^{D23D}	Rab6	No
Rab6^{08323}	Rab6	No
arf79F^{-}	Arf1	No
rab11^{93bj}	Rab11	No
rab11^{j2d}	Rab11	No
rabGGT^{-}	Rab geranylgeranyl transferace	No
shg ^{k03401}	E-cadherin	No
shg^{R69}	E-cadherin	No
dystroglycan^{-}	Dystroglycan	No
dg^{B148}	Dystroglycan	No
ft^{g-rv}	Protocadherin Fat	No
stan^{192}	Atypic cadhein Flamingo	No
l(2)gl^{4}	Giant Larva (cytoskeleton protein)	No
ds^{38k}	Dachsous (Cadherin)	No
cadN2^{-}	Neural Cadherin 2	No
cadN^{-}	Neural Cadherin	No
cadN^{M12}	Neural Cadherin	No
cad74A^{-}	Cadherin74A	No
fat2^{-}	Protocadherin Fat2	No
scrib^{j7B3}	Septate junction Scribble	No

Figure Legends

FIGURE III-1. Molecular lesions of *arl1* alleles: Schematic representations of Arl1 proteins are shown. Wild type Arl1 (180 amino acids) consists of 6α-helices and 6β-stands (WU *et al*. 2003). The $arl1^{4107}$ mutation is predicted to encode a truncated Arl1 protein of 165 amino acids, lacking the C-terminal 15 amino acids; this region is known to form an α-helical (α5) structure in mammalian Arl1. The $arl1^{9.2BC}$ mutation is likely a complete loss-of-function allele, as it was predicted to encode a truncated Arl1 protein of 36 amino acids. The equivalent change for mammalian Arl1 would eliminate the effector and all known protein interaction domains.

FIGURE III-2. Head involution defects in embryos of *arl1* mutants: (A-C) Embryos stained with anti-FasIII antibodies are shown at 40X magnification. (A) A wild type (WT) embryo with normal epithelial pattern. (B) An embryo derived from an $arl1^{9.2BC}/arl1^{9.2BC}$ germ cell exhibits head involution defects (arrow). (C) An embryo derived from an $arl1^{4107}/arl1^{4107}$ germ cell exhibits an abnormal epithelial pattern (head involution defect and body hole are denoted by arrow and square, respectively). (D) An example of normal WT embryonic cuticle is shown. (E) An embryo cuticle derived from an $arl1^{9.2BC}/arl1^{9.2BC}$ germ cell exhibits a head involution defect (arrow). (F) An embryo cuticle derived from an $arl1^{4107}/arl1^{4107}$germ cell with a head involution defect (arrow and large body hole (arrow head). (G) Square inset from (C) is shown at 100X magnification with arrow denoting body hole.

FIGURE III-3. A border cell migration defect in a stage 10 egg chamber expressing $arl1^{Q70L}$-mCherry: Border cells are stained with anti-FasIII and denoted by arrows. (A) In a wild type egg chamber, border cells arrive at the anterior end of the oocyte at stage 10. (B) A

stage 10 egg chamber expressing Arl1-mCherry with normal border cell migration. (C) A stage 10 egg chamber expressing arl1^{Q70L}-mCherry exhibits a border cell migration defect.

FIGURE III-4. Accumulation of Arl1 mRNA in embryos: Arl1 mRNA was ubiquitously distributed in embryos with a higher level in certain tissues. (A) A stage 5 cellularized embryo with Arl1 mRNAs enriched within the cortical layer. (B-E) During stages 6-10, abundant Arl1 mRNAs were detected in the ventral (arrow v), cephalic (arrow c), anterior and posterior transverse (arrows at and pt, respectively) furrows. (F) A late staged 11 embryo that has formed segmentation furrows shows a ubiquitous pattern of Arl1 mRNA accumulation. (G-I) After the completion of gastrulation, Arl mRNAs show enriched accumulation in the brain lobes (arrows) and central nervous system (arrow head).

FIGURE III-5. Subcellular localization of Arl1 proteins in S2 cells: (A) In a S2 cell that is transfected with DNA encoding Arl1-EGFP (Green) and (A') stained with Golgi marker TRITZ-Ceramide (red), (A'') the EGFP-tagged Arl1 is colocalized with the Golgi marker. (B) When the N-terminal 1-17 region was removed (Δ17arl1-EGFP, green), Arl1 lost its fragmented punctate pattern and instead is dispersed throughout the cytoplasm. (C) In a S2 cell transfected with DNAs for Arl1-mCherry (red) and (C') YFP-Golgi (Golgi marker, green), (C'') mCherry-tagged Arl1 is colocalized with the Golgi marker. (D) Myc-tagged Arl1 (red) is colocalized with the YFP-Golgi (green) in S2 cells.

FIGURE III-6. Subcellular localization of Arl1 in oocyte and brain cells: In an oocyte expressing (A) Golgi marker YFP-Golgi (green) and (A') Arl1-mCherry (red), (A'') mCherry-tagged Arl1 proteins exhibit a nice colocalization with the Golgi marker. In brain cells expressing (B) Golgi marker YFP-Golgi (green) and (B') Arl1-mCherry (red), (B'') the mCherry-tagged Arl1 is colocalized with the Golgi marker.

FIGURE III-7. Arl1 can interact with the GRIP domain of Cbs in yeast two-hybrid assay: Interaction of Arl1 proteins, including wild type Arl1 and mutants expected to alter its preference for guanine nucleotide binding (arl1^{Q70L}: GTP; arl1^{T30N}: GDP), with the GRIP domain (GRIP) or the coiled-coil region (CC) preceding the GRIP domain was tested. Arl1 binds to GRIP in a GTP dependent manner, but did not interact with the coiled-coil region preceding the GRIP domain.

FIGURE III-1

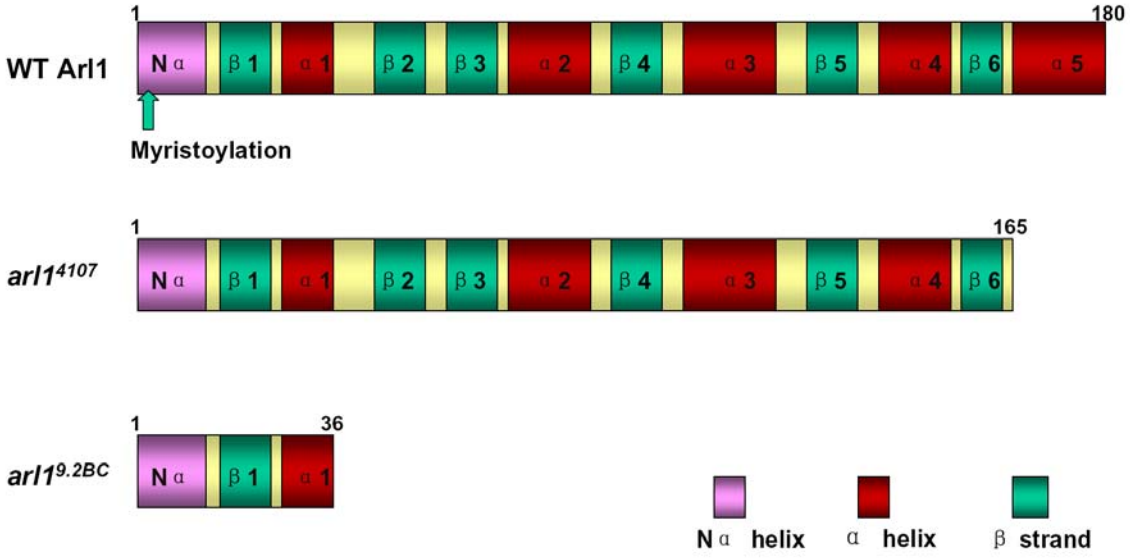

WT Arl1
1
180
N α
β 1
α 1
β 2
β 3
α 2
β 4
α 3
β 5
α 4
β 6
α 5
Myristoylation
arl1^4107
1
165
arl1^9.2BC
1
36
N α helix
α helix
β strand

FIGURE III-2

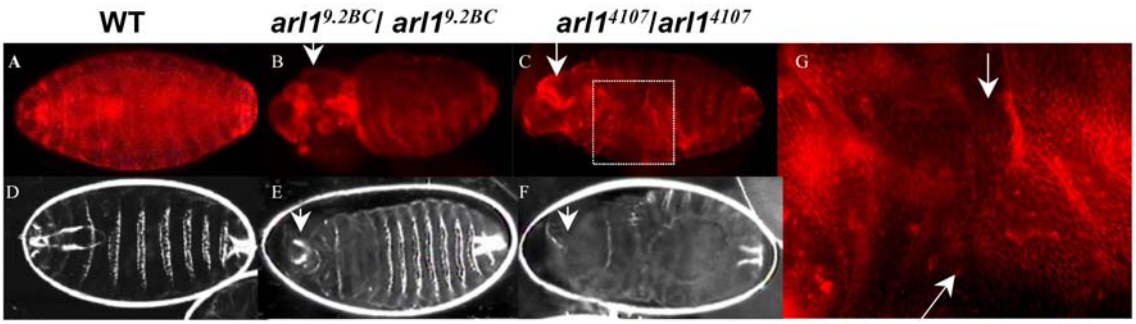


FIGURE III-3

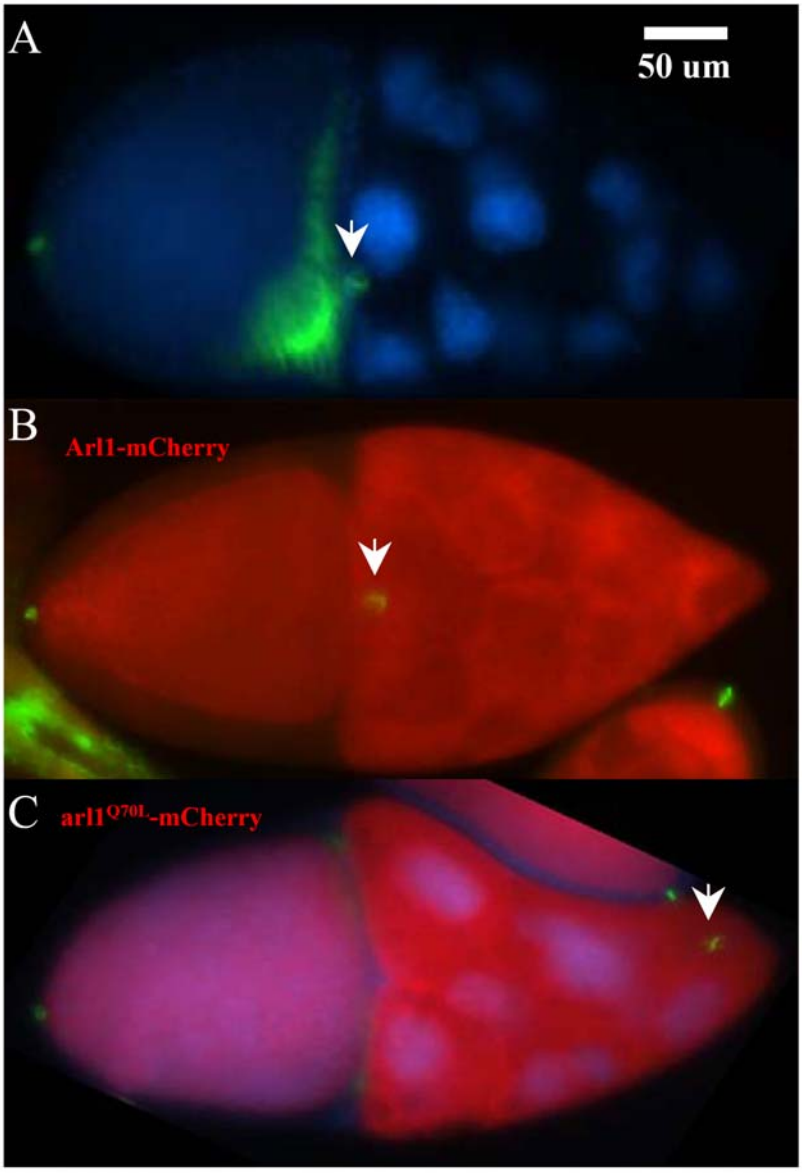


FIGURE III-4

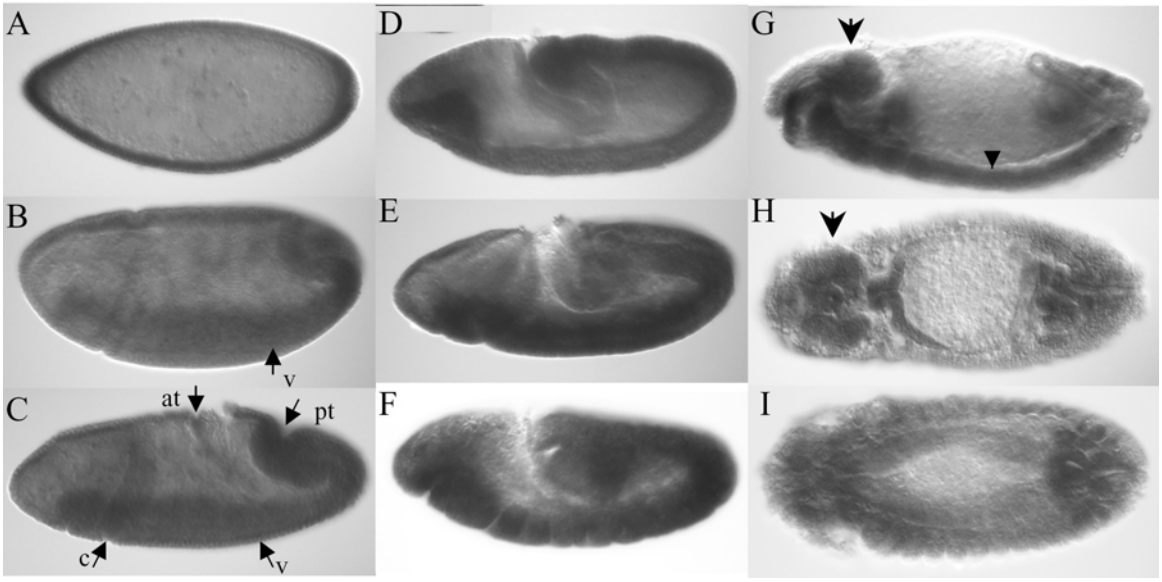


FIGURE III-5

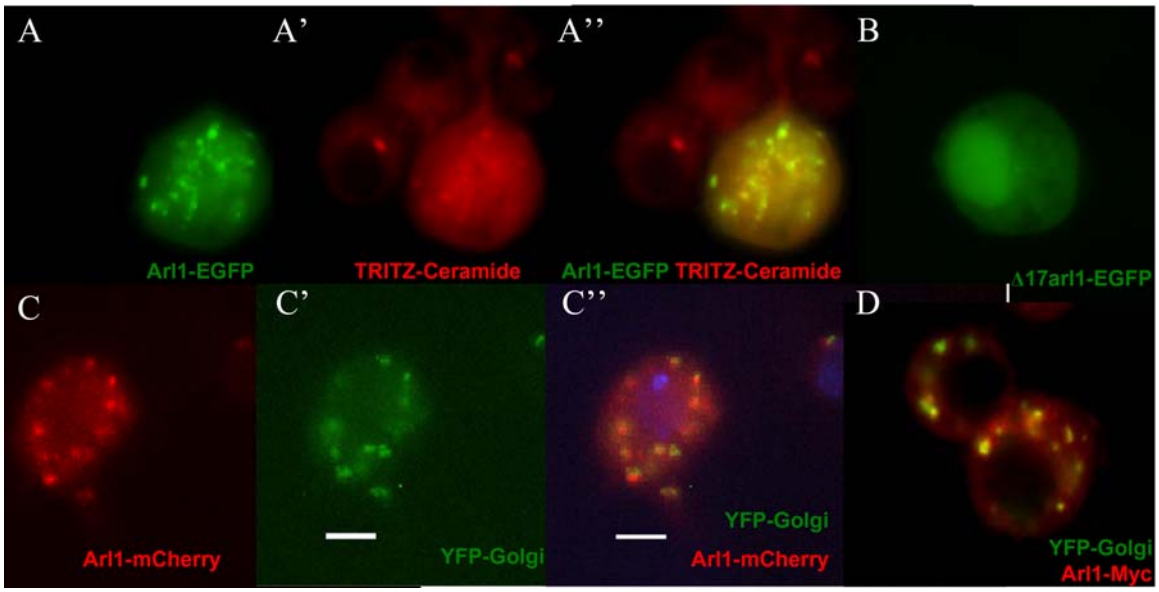

A
A'
A''
B
Arl1-EGFP
TRITZ-Ceramide
Arl1-EGFP TRITZ-Ceramide
Δ17arl1-EGFP
C
C'
C''
D
Arl1-mCherry
YFP-Golgi
YFP-Golgi
Arl1-mCherry
YFP-Golgi
Arl1-Myc

FIGURE III-6

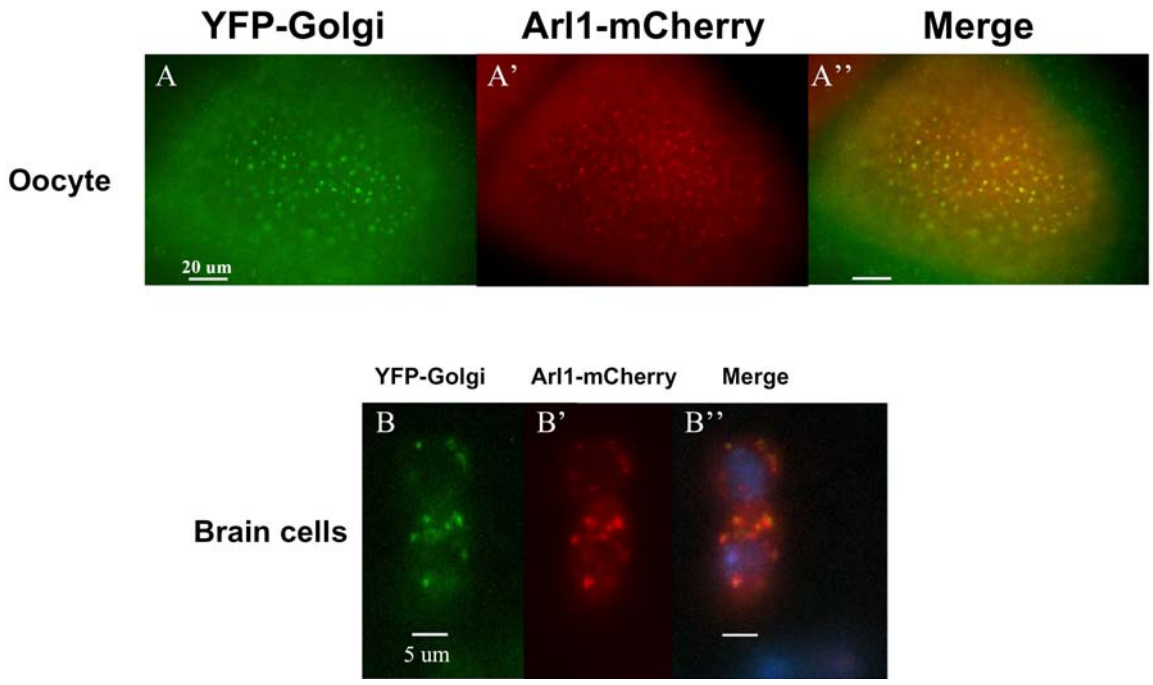
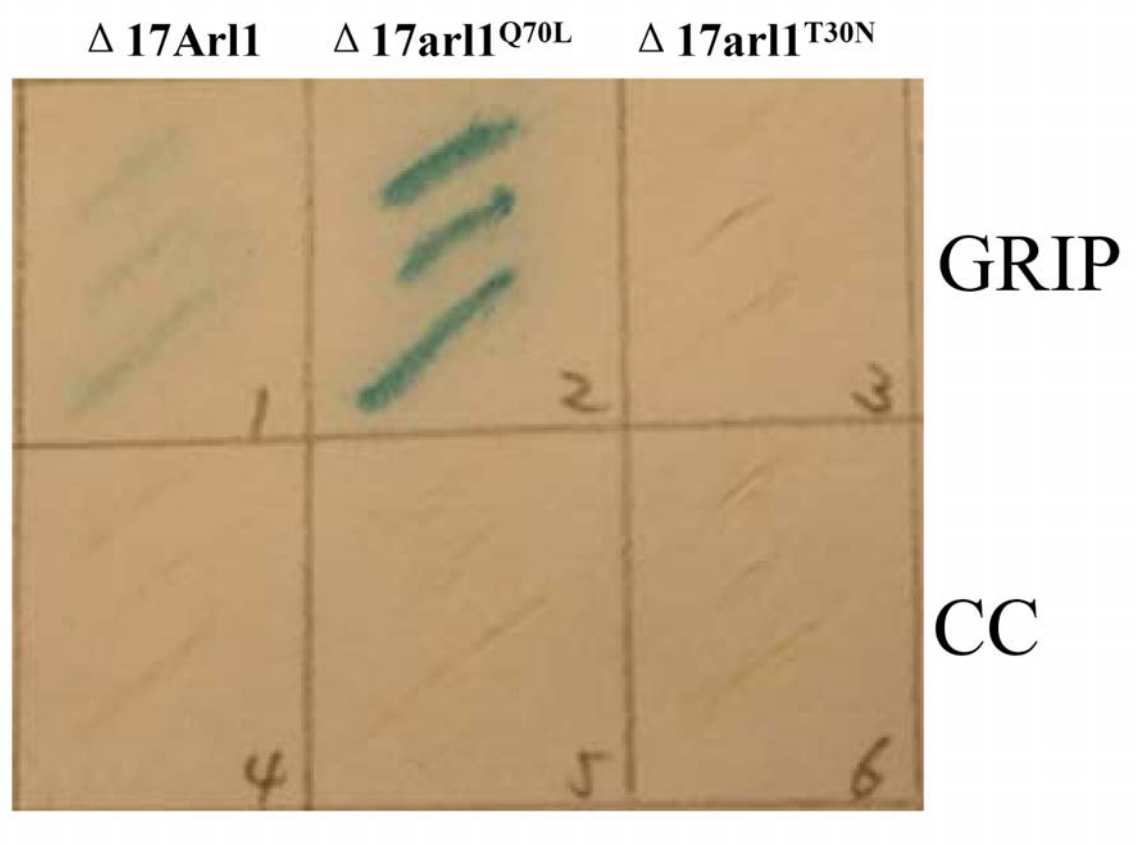

YFP-Golgi
Arl1-mCherry
Merge
Oocyte
A
A'
A''
20 um
YFP-Golgi
Arl1-mCherry
Merge
Brain cells
B
B'
B''
5 um

FIGURE III-7



Contribution of this work

Unless otherwise noted, the experiments in this paper were performed by Jian Ding. The Arl1-mCherry transgenic flies were generated by Yukiko Yamada and Dr. Clark Coffman. Jian Ding and Dr. Ambrosio contributed to the writing, reviewing and editing of this work.

CHAPTER 4. General Summary

The Raf serine/threonine kinases play a key role in ERK signal transduction. However, the mechanisms of Raf regulation are complicated and remain elusive. Clues to regulatory events of Raf were derived from the identification of motifs/sites in these conserved regions.

Raf kinases consist of three conserved regions, CR1, CR2 and CR3. The catalytic kinase portion is located in the C-terminal CR3 region, while CR1 and CR2 represent the regulatory half of the Raf proteins. CR1 contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and is required for recruitment of Raf to the plasma membrane. The Ser/Thr-rich region CR2 has a 14-3-3 binding site, but its functional role is less well defined (WELLBROCK *et al*. 2004). In addition to the three conserved regions, BRaf and DRaf contain an extended N-terminal segment. However, little attention was focused on this region thus far.

Summary and Conclusions

With the general objective of understanding the roles of DRaf's N-terminal segment and the CR2 region: 1) we analyzed the consequences of N-terminal deletion of DRaf both *in vivo* and *in vitro*, and found that the N-terminus of DRaf contains a novel conserved region and can contribute to Toros RTK signaling, potentially by assisting in Ras1 or Rap1 binding; 2) we also performed a yeast two-hybrid screen using the CR2 region as the bait. We identified a small GTPase, Arl1, as a potential binding partner of CR2. Using genetics approaches we have made an effort to understand the functional roles of Arl1 in Drosophila. The studies in this dissertation are summarized here.

Drosophila Raf's N-terminus Contains a Novel Conserved Region and Can Contribute to Torso RTK Signaling: To study the potential function of DRaf's N-terminal residues (amino acids 1-114), we generated transgenic flies expressing full-length DRaf (FL DRaf) or DRaf proteins lacking amino-terminal residues 1-114 ($DRaf^{\Delta N114}$). At ≥ endogenous wild type DRaf levels, maternally expressed $DRaf^{\Delta N114}$ was able to rescue the embryonic terminal defects of $Draf^{11-29}$ mutants. However, when expressed at low maternal levels (~1/4 endogenous wild type DRaf level), terminal defects, as well as, abnormal posterior *tll* expression pattern (<13% EL) was observed more often for embryos that inherited truncated $DRaf^{\Delta 114}$ rather than full-length DRaf proteins. This finding suggested that deletion of the N-terminual residues reduces DRaf's signal potential in the Torso RTK pathway. Furthermore, in the sensitized tor^{RL3} background, the ectopic gain-of-function effects of tor^{RL3} allele were more significantly enhanced by over-expressed FL DRaf, compared to $DRaf^{\Delta 114}$. Together, these data indicate that the extended N-terminal segment could contribute to Torso RTK signaling.

Accordingly, FL DRaf also exhibited higher signal potential than $DRaf^{\Delta 114}$ proteins in a trk^{1} background, in which a small amount of Torso signal activity may exist. Over-expression of FL DRaf but not $DRaf^{\Delta 114}$ partially restored the posterior terminal defects (A8 denticle belt) in some embryos from trk^{1}/trk^{1} mothers, again implying that the N-terminus can participate in Torso RTK signaling. However, in the tor^{XR1}/tor^{XR1} background with complete loss of receptor activity, expression of neither FL DRaf nor DRaf restored terminal structures (A8 denticle belt). Thereby, the N-terminal region's contribution to the RTK pathway appeared to be dependent on upstream receptor activity, suggesting that it may play a role(s) in transmission of the signal.

We analyzed the amino acid sequence of DRaf's N-terminus and identified a novel region (CRN) conserved in BRaf proteins. Circular dichroism analysis indicated that DRaf's N-terminus (amino acids 1-117) including CRN (amino acids 19-77) is folded *in vitro* and has a high content of helical secondary structure as predicted by proteomics tools. In yeast two-hybrid assays, stronger interactions between DRaf's Ras binding domain (RBD) and the small GTPase Ras1, as well as Rap1, were observed when CRN and RBD sequences were linked. This is consistent with the results obtained by Fischer *et al.* (2007) for BRaf.

Together, our in vivo and in vitro assays suggest that DRaf's extended N-terminus can assist in its association with the upstream activators (Ras1 and Rap1) *in vivo* and thus, play a regulatory role(s) in DRaf signal transmission potentially through a CRN-mediated mechanism(s).

The N-terminus of DRaf seems to participate in Ras1 and Rap1 binding, but as an isolated protein fragment cannot directly interact with Ras1 or Rap1. Furthermore, the effects of the N-terminal residues on Ras1 or Rap1 binding *in vitro*, as well as on Torso RTK signaling *in vivo* appears to be subtle. However, the minor contribution of the extended N-terminus could have important biological implications. We propose that tight association with Ras1 or Rap1 through RBD and CRD regions is required and sufficient to initiate the activation of DRaf, while minor adjustments/regulation of the interaction by CRN could optimize signaling potential and reduce variability. Thus, the extended N-terminus including CRN may play a role(s) as one element in a multi-domain effort to promote DRaf's interaction with Ras1 and Rap1, participating and assisting in its regulation to reliably attain maximal signal output.

Initial characterization of Drosophila Arl1: We initiated our study on Arl1 based on the hypothesis that Arl1 may be involved in DRaf signaling, since Arl1 can interact with the CR2 region of DRaf in our Yeast two-hybrid assay. Three different *arl1* alleles were analyzed using classical genetic methods. However, we did not detect DRaf relevant phenotypes for Arl1 mutants.

We found most homozygous *arl1*$^{-}$ mutants proceeded through embryogenesis and died during larval-pupa stage. Cuticles of unhatched embryos or *arl1*$^{-}$*/arl1*$^{-}$ larvae appeared to be relatively normal. Thereby, zygotic Arl1 may not be essential for the development of embryos.

Next we generated females with *arl1*$^{-}$*/arl1*$^{-}$ germ cells to understand Arl1's maternal role. Most embryos lacking maternal Arl1 activity appeared to be relatively normal, although ~6% of them exhibited head involution defects. When crossed with *arl1*$^{-}$/+ males, the hatching rates of eggs from females with *arl1*$^{-}$*/arl1*$^{-}$ germ lines were not significantly altered, implying removal of paternal Arl1 in half of the eggs did not appear to affect their embryogenesis. It seems that both maternal and zygotic Arl1 is limitedly required during embryo development.

The head involution defects we observed, although at low penetrance, suggest Arl1 can be involved in cellular morphogenesis. Considering Arl1's potential role(s) in intracellular transport, we tested for genetic interaction between *arl1* alleles and mutations in numerous genes encoding membrane-localized adhesion molecules. These have been shown or are thought to function in cellular morphogenetic movement due to their dynamic presence on the cell surface, which is regulated by endocytosis and exocytosis (EDELMAN 1985;

GUMBINER 1996; BRYANT and STOW 2004; VANHOOK and LETSOU 2007). However, no additive or synergistic effects were seen.

The mild phenotypes observed in *arl1* mutants could be due to the functional redundancy of Arl1 with other small GTPases, including Rab proteins. However no genetic interaction, with the limited number of candidates that we tested, was detected. Therefore, there might be other proteins that function redundantly with Arl1. Together, our analysis implies that it is difficult to dissect Arl1's roles using classical genetic methods to study these *arl1* alleles.

Next, we employed an over-expression strategy to characterize Arl1's function in Drosophila. We found over-expression of arl1^{Q70L}-mCherry in germ cells reduced female fertility and ~8% of stage 10 egg chambers of these females exhibited delay in border cell migration. Considering Arl1's potential functions in intracellular transport, we proposed that the border cell migration defects upon over-expression of arl1^{Q70L}-mCherry might be due to dysregulation of the adhesion molecules in nurse cells. However, none of the mutations in adhesion molecules we tested showed additive or synergistic effects when arl1^{Q70L}-mCherry was over-expressed in nurse cells.

We also examined Drosophila Arl1's subcellular localization. Epitope-tagged Arl1 proteins were colocalized with golgi markers both *in vitro* for Drosophila S2 cells and *in vivo* for the oocyte and brain cells. This is consistent with the studies by others in yeast and mammalian cells. We also found that the mRNA of Ar11 ubiquitously accumulates in embryos with higher levels observed for certain tissues.

Together, our genetic analysis of three different *arl1* alleles suggested that Arl1 could function in cell morphogenesis. Although Arl1 was found to interact with DRaf's CR2 region

in our yeast two-hybrid assay, it seemed that mutations in Arl1 did not alter DRaf signaling. While an essential gene, Arl1 plays limited roles during Drosophila embryogenesis, likely due to the functional redundancy with other genes. Our studies provide the basis, which may be helpful for further characterization of Arl1's function.

Suggestions for Future Research

Study of DRaf or BRaf's N-terminus:

1) The N-terminus of DRaf appears to participate in Ras1 binding, however, the details of this process is still unclear. Structural analysis of the complexes (*i.e.*, NRBD-Ras *etc.*), may provide important clues and help to understand the molecular mechanism(s) by which CRN assists in Ras-Raf interaction.

2) The CRN region contains several conserved putative phosphorylation sites, which may play regulatory roles in DRaf or BRaf signaling. Studying the consequences of point mutation on DRaf's signal potential or Ras binding would provide important insights into the functional roles and mechanisms of this N-terminal region.

3) Compared to ARaf and CRaf, BRaf is more liable to oncogenic mutation, likely due to its higher basal activity. The negative charged D447-D448 motif (corresponding to E420-E421 in DRaf) preceding the kinase domain in BRaf mimics the constitutive phosphorylation in this region (MASON *et al*., 1999). This may account for its high basal activity. Fischer et al. (2007) reported that CRaf's activity was elevated ~25% by the addition of the first 98 amino acids of BRaf to its N-terminus, implying that the extended N-terminus may contribute to BRaf's high basal activity, too. One could test if there is additive or synergistic interaction between the N-terminal residues and the negative charged phophomimetic motif. Particularly,

comparing the activity of $DRaf^{\Delta N114,V420V421}$, $DRaf^{V420V421}$, $DRaf^{\Delta N114}$ and FL DRaf ($BRaf^{\Delta N98,V447V448}$, $BRaf^{\Delta N98,V447V448}$, $BRaf^{\Delta N98}$ and FL BRaf) would be helpful to extend our understanding of DRaf or BRaf's basal activity, as well as, the liability to oncogenic mutations.

4) $BRaf^{V599E}$ accounts for ~90% oncogenic BRaf mutations in human cancers (SRIKALA *et al.* 2005). $BRaf^{V599E}$ is a constitutive active mutant. Although its gain-of-function effects seem independent of Ras binding, it is still possible that presence of the extended N-terminus may contribute to its constitutive activity. One could test if the deletion of the N-terminus affects the oncogenic activity of $BRaf^{V599E}$.

Further characterization on Arl1:

1) While an essential gene, Arl1 seems to play limited roles in Drosophila embryogenesis. Only mild defects were observed in Arl1 mutants. Lack of significant phenotypic information is likely due to its functional redundancy with other genes. We tested whether there were additive or synergistic effects between *arl1* alleles and mutations of several candidate genes, which may act redundantly with Arl1. However, due to the difficulty of generating complex genetic backgrounds using classical methods, we did not resolve the issue of functional redundancy because our genetics interaction assays appeared not sensitive enough. NIH has just begun to support a TRiP (Transgenic RNAi Project) project to develop the UAS-RNAi system in a genome-wide scale. One could also combine RNAi sequences of several different genes (Arl1, Rab2, Rab6, *etc.*) and simultaneously deplete their functions in flies. This would be very helpful for characterization of Arl1's functions.

2) Identification of the components/cargo of Arl1 complex/trafficking machinery using proteomic approaches could also be helpful to characterize its developmental roles. Particularly, one could co-precipitate or pull-down the proteins of Arl1 complex from cellular lysates, and using mass spectrum analysis to identify the components. A combination of these proteomics approaches could allow the *in vivo* genetic analysis of Arl1 more promising.

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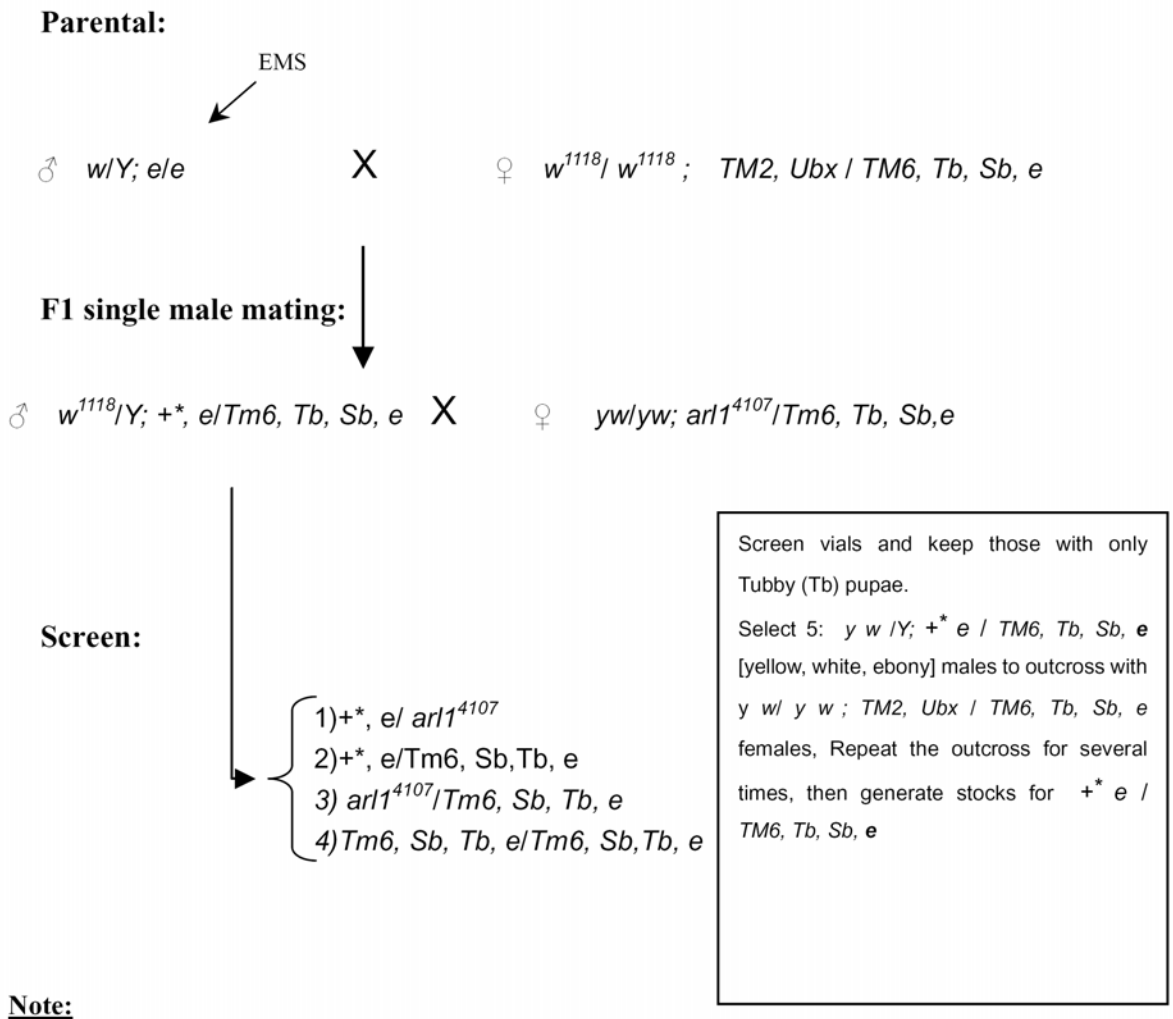
APPENDIX

Appendix 1. Genetic Screen for *arl* alleles: EMS mutagenesis



Note:

Double *Tm6* balancer→ lethal

X chromosome markers:

w= white eyes, recessive

w^{1118}= white eyes, recessive

y= yellow wings/body, recessive

3rd chromosome markers:

Ubx=large halters, dominant

Sb=short bristles, dominant

Tb=Short body, dominant

e=dark body, recessive

Appendix 2. List of primers

Primer	Sequence
EcoRIRaf-F	CGGAATTCACCATGTCCAGCGAGTCCTCCAC
EcoRICR1-F	CGGAATTCACCATGCACCACGGCAACGATC
EcoRICR2-F	CG *GAA TTC* ATG CTG TGC CAG CCC TTT C
EcoRICR3-F	CG *GAA TTC* GTG TGC ATC AAC AAC ATT CG
EcoRIΔ17Raf-F	CGGAATTCATGGCCGAGGAGCTGCAC
EcoRIΔ77Raf-F	CGGAATTCATGAACTCGCAGGACCAGC
BamHIRaf-R	CGCGGATCCCGCTGTTTAGATATTCCCAG
BamHICR1-R	CG GGATCC GAAAGGGC TA GCACAGCATC
BamHIRBD-R	CGG GAT CCT TAT TCC AGC AGC CTG ACA AAG ATC
BamHICR2-R	CG GGATCC GTT GTT GAT TCA CACATTTGG
BamHIN-R	CGGGATCCTCAATGTACTCGCGCCAATTGCCGTTG
C110-F	GGC CCT GAA ACT CCT GCA ACT AAC GCC GGA TAT G
C110-R	CAT ATC CGG CGT TAG TTG CAG GAG TTT CAG GGC C
NTE-F	GAATACCAGGAGTTGGAATCCAAGCTCCACGAACTG
NTE-R	CAGTTCGTGGAGCTTGGATTCCAACTCCTGGTATTC
NTV-F	GAA TAC CAG GAG TTG GTA TCC AAG CTC CAC GAA CTG
NTV-R	CAG TTC GTG GAG CTT GGA TAC CAA CTC CTG GTA TTC
EcoRIRas-F	CG GAA TTCATGACGGAATACAAACTGGTC
BamHIRasΔ-R	CG GGA TCC TA TCTACAATTCGGCTTGTTC
EcoRIRap-F	CGG AAT TCA TGC GTG AGT ACA AAA TCG TG
BamHIRapΔ-R	CGG GAT CCT TAT AGG GAC TTT TTC GGC TTC
EcoRI1433-F	CGGAATTCATGGCGACAGTCGATAAGG
BamHI1433-R	CG GGA TCC TAAGTTGTTTTGGTTAGTTGTC
ClaIKSRCA-F	CC AT CGA TCC AGGATGAGCAGCAACAAC
XhoIKSRCA-R	CCCTCGAG CTCTTATAGCCGCATCTTCG
XhoIKSRCC-R	CC CTC GAG TTA CGC AGT ACCGCTCTCCAG
XhoIKSRN-R	CC CTC GAG TCA GCC ACT GCT CGT CAC
HindIIIArl-F	AAGCTTCGCTCAACTTTGGCAGATAC
HindIIIΔ17arl-F	CG AAGCTTA CCA TGC GCA TCT TAA TCC TGG
BamHIArl-R	CG GGATCC GA AAG TAT CTA CTT GCG ACT C
KpnIArl-F	GG*GGTACC*ACACGCTCAACTTTGGCAGATAC
XbaIArl-R	GC TCT AGA TGA TGA CTT CCG ACT CTG C
XbaImChe-F	GATCTAGAGCAACCATGGTGAGCAAGG
AgeImChe-R	TCACCGGTTTCTTACTTGTACAGCTCGTC
EcoRIArl-F	CG GAA TTC ACC ATG GGT GGG GTG CTC
BamHIChe-R	CG GGA TCC TTCTTACTTGTACAGCTCGTC
EcoRIΔ17arl-F	CGG AAT TCA CCA TGC GCA TCT TAA TCC TGG
BamHIArlS-R	CG GGATCC GA AAG TAT CTA CTT GCG ACT C
T30N-F	GA CGG CGC CGG CAA GAA CAC GAT CCT CTA CAG
T30N-R	CTG TAG AGG ATC GTG TTC TTG CCG GCG CCG TC
NdeICbsCC-F	CC CAT ATG GACGAAACCATAATGCAACTG

BamHICbsCC-R	CG GGA TCC GTGTTTCAAATATTGAAAATTAAC
EcoRIGrip-F	CG GAA TTC GTTAATTTTCAATATTTGAAACAC
BamHIGrip-R	CG GG ATC CTA CAA TGT GTC GTG TAG CAG C
tll-F	TCGGAGGGTTCACCAGAC
tll-R	GAGATGAGGCGCACAATGG
en-F	AATGGCCCTGGAGGATCG
en-R	CCGTCAGAAGGAGTAACCC
P1-F	GCGCCA GCACTAGACAAAG
P1-R	GAAATAGCACGAATTGAGATTAG

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